

EVALUATION OF SHORT-TERM TESTS FOR TOXICITY AND MUTAGENICITY WITH SPECIAL REFERENCE TO MERCURY AND SELENIUM

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"The birds, for example - where had they gone?"

(Rachel Carson)

TO BERTIL

ANNE-LI

MAGNUS

ODDVAR

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This thesis is based on the following papers:

- I. Fiskesjö, G. 1969. Some results from Allium tests with organic mercury halogenides. - Hereditas 62:314-322
- II. Fiskesjö, G. 1970. The effect of two organic mercury compounds on human leucocytes in vitro. - Hereditas 64:142-146
- III. Fiskesjö, G. 1971. The effect of two mercury compounds on lysogenic E. coli K39(λ). - Hereditas 69:135-138
- IV. Fiskesjö, G. 1979. Two organic mercury compounds tested for mutagenicity in mammalian cells by use of the cell line V 79-4. - Hereditas 90:103-109
- V. Fiskesjö, G. 1979. Mercury and selenium in a modified Allium test. - Hereditas 91:169-178
- VI. Fiskesjö, G. 1974. Two types of constitutive heterochromatin made visible in Allium by a rapid C-banding method. - Hereditas 78:153-156
- VII. Fiskesjö, G. 1975. Chromosomal relationships between three species of Allium as revealed by C-banding. - Hereditas 81:23-32
- VIII. Fiskesjö, G. 1981. Benzo(a)pyrene and N-methyl-N-nitro-N-nitrosoguanidin in the Allium test. - Hereditas 95: 155-162

References to these papers will be made by the above Roman numerals



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INTRODUCTION

The mercury problem

The mercury story in Sweden developed parallel to the increased trend of using chemicals in America. The misuse of chemicals initiated the work of Rachel Carson "Silent Spring" (1962), which is a scientific painting demonstrating the connection between the spreading of chemicals and the destruction of nature and the risk for human health. In Sweden, reports on the increasing environmental problems and risks due to misuse of chemicals were given by Palmstierna (1967) and 1959 by Ehrenberg and Gustafsson (published 1970). That chemicals may produce inheritable changes was first shown experimentally already in 1944 (Auerbach and Robson).

From about 1940 to 1965, alkyl mercury was commonly used as a fungicide, and different forms of mercury were included in the waste from industries, power plants and other sources (National Swedish Environmental Protection Board 1973). The first mercury alarm was given in Sweden by Borg (1958) and later by Borg et al. (1965) when it was found that mercury caused the death of many birds. This led to the prohibition for use of alkyl mercury. Restricted use of the less stable alkoxy alkyl mercury was still allowed by the Poison Board (Giftnämnden) (Svensk Författningssamling 1965), and the restrictions caused a considerable decrease in mercury waste.

However, the mercury problem is far from solved. For instance, the mercury poisoning of humans in Iraq (Bakir et al. 1973), which was on an even larger scale than the Minimata catastrophe 1953 (for review see d'Itri 1972), shows that alarms and knowledge spread very slowly.

In Sweden new alarms have come recently concerning the increased mercury content in many lakes (National Swedish Environmental Protection Board 1982); an increase that partly depends on the water acidification affecting the release of sedimented mercury in the lakes, and partly on the amount of airborne mercury.

In the light of eventual antagonistic effects of selenium compounds, which will be discussed below, the research on mercury problems may turn into a new direction.

How dangerous are the small doses?

In a review on the use of mercury in Sweden (Landell 1968) are also included reports from the discussions that led to recommendations for restricted consumption of mercury-contaminated food. The maximum limit value, which is still valid (National Swedish Protection Board 1982), was set to 1 mg/kg.

Based on the knowledge that mercury is accumulated and stored in living organisms (e.g. Boëtius 1960, Hannerz 1968, Ekman et al. 1968; for review see Nordberg and Skerfving 1972), and also considering the results in the present papers (I - V, VIII) on biological damage effects from lower concentrations, the limit value 1 mg/kg food seems to be too high. In addition, the biological half time of mercury in humans is 70 days (Bergmark 1974, Clarkson 1977); a relatively long time with the possibilities of reactions between the mercury and cells in the human body.

To indicate the relative toxicity of different compounds, LD50 values (the concentration which kills 50% of the test organisms) have been commonly used. Concerning the risk for genotoxicological damage effects from small doses, the LD50 values are not sufficiently informative. Therefore, effects of small doses by long time exposure should instead be considered in more sensitive short term test systems, where for instance the parameters for mutagenicity or chromosome abnormalities may be evaluated. These parameters are highly correlated to each other and also to carcinogenesis (e.g. Ehrenberg 1974, Huberman 1975, deSerres 1976a).

A widely discussed question is if threshold values in vivo exist or not. The assumed dose response curves for chemicals (Maugh 1978a) are similar to those for effects of radiation (Barnaby 1980). Many scientists now argue that complex metabolic routes (e.g. repair systems) can minimize the danger from many carcinogens. The threshold hypothesis implies the assumption that there is a no-effect dose below which induction of cancer cannot occur or occurs with an extremely low probability (Maugh 1978a).

However, a multiple-hit theory is also considered (Maugh 1978a), involving a first mutation step and a later promotion of the event to phenotypic expression. Further work with short-term test systems may be elucidative as to the connection between effects of small doses and the risk for genotoxic changes in whole organisms.

Which test systems should be used?

Evaluation of new and established test systems are currently undertaken in different laboratories. In Sweden certain guidelines were proposed by a meeting already in 1972 (Royal Swedish Academy of Sciences 1973), and recently 51 tests (from more than 100 in use) have been confirmed for use within the OECD countries (OECD 1981).

Concerning the decisions for allowing the use of chemical compounds, one single test is not enough. Test batteries, which should cover different types of metabolism in different organisms, have been proposed (e.g. Royal Swedish Academy of Sciences 1973, Committee 17, 1975). According to Maugh (1978b) the variety of test systems could be broken down into 4 broad classes: 1. Tests with microorganisms, 2. Tests with intact organisms, 3. Tests for genetic damage and mutations in cultured mammalian cells, 4. Tests for in vitro transformation.

The test systems used in the present work (I - V, VIII and this paper) represent three of the above mentioned groups. Applying four different test systems, the effects of especially some mercury and selenium compounds will be compared.

TEST METHODS USED IN THE PRESENT STUDY

The purpose of the comparison of several test systems was to acquire increased knowledge about the reliability of these tests. The applied test methods are discussed in detail in papers I - V and will be shortly commented upon below.

A method for chromosome banding in Allium cepa, one of the test materials, is documented in papers VI and VII, and in paper VIII this banding method is tried in combination with certain chemicals. Paper VIII, though, is mainly a study on the application of the Allium test system on water insoluble compounds, with the main purpose to investigate the presence of a MFO(mixed-function oxidases)-system in Allium.

Tests with Allium (I, V, VIII)

Allium cepa was used already by Nemec (1904) to study chromosome reactions in root tips of certain chemicals. The use of Allium cepa as a test system was introduced by Levan (1938) when the effects of colchicine were investigated. Since then, the Allium test has been frequently used (e.g. Levan and Östergren 1943, Levan 1945, Kihlman 1966, Fiskesjö 1969(I), 1975, 1979a, 1981a, 1981b, 1981c, Fiskesjö et al. 1981, Ramel 1969, Kubiak 1971, Bielecki 1974, Härtley-Asp 1976, Linnainmaa 1978, 1979).

The original form of the test comprises the outgrowth of root tips in fresh water followed by treatment with test chemical solutions. For the test of complex mixtures, as for instance river water, a modification of the test has been developed (Fiskesjö 1975). Here, a number of replicates of test onions are placed directly in the test liquids; a method which has proven to be useful for known chemicals as well (e.g. V, VIII). In the microscopic investigations the usual cytological parameters are studied, including for instance mitotic index, chromosome aberrations, aneuploidy and c-mitosis (colchicine-like disturbances of the mitotic process).

Tests with human lymphocytes (II)

The conventional blood culture technique (Moorhead et al. 1960) was used to study the effects of mercury compounds on lymphocytes. In order to avoid the counteracting action of the culture medium components cysteine (S-H) and cystine (S-S) to mercury, the treatment has been performed in a salt solution devoid of cysteine and cystine.

This study (paper II) was done before the introduction of chromosome banding techniques. The orcein squash technique was sufficient for the study of different parameters such as c-mitosis and chromosome breaks and also for the determination of a threshold value for toxicity and mitotic abnormalities in this short-term test.

Tests with microorganisms (III)

Two strains of E. coli (K39(λ) and K49) were used as test material. The method was a modification of the procedure to induce lysis in lysogenic bacteria by UV-light (Weigle and Delbrück 1951); instead of UV-light a 1 h treatment with certain mercuries were performed. Toxic effects (inactivation of bacteria and phages) as well as effects directly on DNA (induction of phages) were used as parameters.

Tests with mammalian cells (IV)

The test material, the Chinese hamster cell line V 79, was isolated by Chu et al. (1969) and has since then become a standard cell line for the study of cellular mutagenesis (Arlett 1977); for review see Bradley et al. 1981).

The methods follows in detail the scheme outlined by Huberman (1975), comprising tests for toxicity and mutagenicity.

The two genetic markers aza and oua are used to select mutants. In both loci forward mutations will cause changes from sensitivity to resistance (to 8-azaguanine, aza^R , and ouabain, oua^R), and both loci are shared by several types of mammalian cells (see paper IV for references).

Two methods for selection of mutants have been in use: The selection in situ (only one spreading of the cells directly

after treatment) has been practiced by several authors (Chu et al. 1969, Huberman 1975, Huberman and Sachs 1974, 1976, Arlett 1979); whereas the respreading method (repeated spreading of the growing mutants) is thought to imply better possibilities for the establishing of the mutants (Fox 1975, Abbondandolo et al. 1976, van Zeeland and Simons 1976, Knaap and Simons 1977, Arlett 1979). Concerning the oua-mutants, Arlett (1979) claims that the respreading method is not suitable because of the sensitivity of these mutants to trypsin.

To my experience, the in situ method gave the best results for both aza- and oua-mutants.

When using human lymphocytes as a test system (paper II), the mercury treatment was performed in a salt solution to avoid the risk of Hg/medium-interaction. Since preliminary results with V 79 cells showed clear effects of mercury even in lower doses than for the lymphocytes, and since the 3 h duration of the treatment may cause a decrease in cell survival, Hg/cysteine and Hg/cystine interactions in the medium were ignored and the treatment was performed in culture medium.

Chromosome banding of Allium (VI, VII and VIII)

The introduction of chromosome banding methods in the early 70-ies resulted in better possibilities to make a more detailed study of chromosome structure and damage effects of chromosomes. A slight modification of the C-banding method for rye (Sarma and Natarajan 1973) was successfully used for C-banding of Allium (VI, VII), and this method was tried in combination with treatments with certain carcinogens (VIII).

MERCURY IN DIFFERENT TEST SYSTEMS

Mercury is an element non-essential to man. The toxicity of mercury is well known and its chemical properties, as well as occurrence, use and effects on different organisms are extensively reviewed (d'Itri 1972, Friberg and Vostal 1972, Laveskog et al. 1976, WHO 1976, Panel on Mercury 1978, National Swedish Environmental Protection Board 1978).

Considering the in vivo situation there is a slightly varying disposition of different cell types to react with and to store mercury (e.g. Ekman et al. 1968, Nishigaki and Harada 1975, Suckcharoen et al. 1978). Thus, certain differences in response between different test systems could be expected. Östlund, (1969), however, found that the uptake of mercury in different organs was approximately proportional to the dose given.

The two organic mercury compounds tested for comparison in four different test systems were methyl mercury chloride (MMC) and methoxyethyl mercury chloride (MOEMC). The most typical action of these metalorganic compounds is that they bind to sulfhydryl groups in the proteins (Iversen et al. 1979). It is also known that mercury tends to bind to the purines in DNA (Freese 1971). Thus, these agents might be expected to act in a more or less similar way.

The Allium test (I)

My first attempt to approach the problem of genotoxic effects of environmental chemicals was the application of the Allium test (original method) on a commercial fungicide (Betoxin) containing certain mercury compounds. The subsequent studies on the effects of pure mercury halogenides on Allium (I) confirmed other results (Levan 1945, 1951, Fahmy 1951, Ramel 1969) showing the typical c-mitotic effect of the mercuries tested (methyl mercury chloride, MMC; ethyl mercury chloride, EMC; methoxyethyl mercury chloride, MOEMC; buthyl mercury bromide, BMB).

A low incidence of chromosome breaks was noticed after certain treatments.

Threshold values for cytotoxic effects in this system were determined; the higher the molecular weight the lower were the threshold values (Table 1).

Table 1. Lowest concentrations ($\times 10^{-6}$ M) showing microscopical disturbances after 4 h treatment (From I, Table 2)

MMC	EMC	MOEMC	BMB
2	0.2	0.2	0.05

An interesting macroscopic reaction after treatment with certain concentrations was the "crocket hook" reaction earlier described by Ehrenberg et al. (1949). According to Levan (personal communication) this effect may be a typical heavy metal effect, and in a later study (Fiskesjö 1982a), the crocket hook reaction was observed after treatments with Cd- and Be-salts.

The occurrence of aneuploidy after Se/Hg-treatment will be discussed later (p.17).

The lymphocyte test (II)

By the study of human chromosomes after mercury treatments (MMC and MOEMC) in the in vitro lymphocyte test (II), the results were about the same as in the case of Allium. Both the mercuries were highly toxic and yielded in some concentrations an extremely high frequency of c-mitosis. A certain low incidence of chromosome breaks was noticed; the frequency was estimated to be considerably less than 1% of the mitoses.

Chromosome aberrations have been recorded in human lymphocytes after in vivo exposure, although the statistical significances of structural and numerical changes were not confirmatory in the studies of Skerfving (1970, 1972) and Verschaeve et al. (1976).

Tests with lysogenic bacteria (III)

The very low frequency of chromosome breaks in the two chromosome systems tested (I and II) raised the question if it should be possible to measure any direct effect on bacterial DNA in tests with lysogenic bacteria *E. coli* K39(λ) and induction of the phage λ (III). This proved not to be the case. However, the threshold values for toxic effects (inactivation of bacteria and phages) were close to those for toxic effects in the two systems tested earlier.

The reason why no induction of phages could be observed in these experiments may be either that the mutagenic effects of the mercury concentrations used are too low, or that mutagenic effects in eucaryotic cells cannot be correlated to induction of phages in procaryotic cells under the present experimental conditions.

The V 79 test system (IV)

When the two mercuries MMC and MOEMC were tested in the V 79 test system (IV), interestingly enough lower threshold values were obtained and certain mutagenicity effects were observed below the threshold for toxic effects. Though, the lower toxicity level for the V 79 cells may depend on the longer time of treatment (3 h compared to 1 h for the lymphocytes and for the microorganisms).

This means that in all the four test systems and in all the five organisms tested (I - IV), there is a similar sensitivity to the mercury compounds tested (Table 2). Since mercury in organic compounds is closely bound to the organic part of the molecule (Jensen and Jernelöv 1971), only the molarity for the whole compound is given.

Table 2. Threshold values for effects of MMC and MOEMC in four different test systems. The lowest concentrations leading to growth disturbances are given in molarity ($\times 10^{-6}$) and in (ppm). (Partly from IV, Table 3)

Test chemicals	Toxicity tests					Mutagenicity test
	Bacteria	Phages	Allium	Lympho- cytes	V 79	V 79
MMC	2 (0.5)	2 (0.5)	2 (0.5)	1 (0.25)	0.8(0.2)	0.4 (0.1)
MOEMC	4.2(1.25)	1.6(0.5)	1.6(0.5)	1.6(0.5)	0.33(0.1)	0.16(0.05)

The fact that mutagenic effects are manifested in concentrations below the threshold for toxic effects (Table 2) means that mutagenicity testing may be the more sensitive tool to detect biological damage effects of low doses.

Mercury compounds have been shown earlier to be mutagenic in Drosophila. Thus, a slight increase in the frequency of X-linked recessive lethals was reported by Ramel (1972), and more significant increases in X-linked recessive lethals have been observed after exposure to mercury-p-toluene-sulphanamide (Mathew and Al-Doori 1976).

The present work on V 79 cells (IV) is the first to show mutagenic effects of mercury compounds in mammalian cells. There is a clear dose-response effect within a very narrow range of concentrations (IV, Fig. 2). In this respect, tests with mammalian cells are somewhat inferior to microbial tests. In addition to the relatively fewer mutation events scored in mammalian cells, mutation induction here is frequently not observable until significantly toxic concentrations of mutagens are reached, and the dose-response is over a very narrow dose-range (Hollstein et al. 1979).

MERCURY COMBINED WITH SELENIUM

The Allium test (V)

Data from animal studies have suggested that selenium has a protecting effect against methyl mercury poisoning, but the metabolic function of selenium and also the biochemical mechanism for selenium-methyl mercury antagonism is not yet understood (O'dell and Campbell 1972, Oehme 1972, Panel on Mercury 1978).

It is known that mercury acts by binding to sulfhydryl compounds (Wachtmeister 1971), and Sugiura et al. (1976) have shown that the selenohydryl compound binds methyl mercury 100 times more tightly than the sulfhydryl group. However, Tsen and Collier (1959) found no support for the hypothesis that selenite can be an inhibitor of sulfhydryl enzymes, and supposed that selenium interferes with certain co-factors in intermediary metabolic pathways in the cell.

When Se was shown to be antagonistic to methyl mercury in animal experiments (Parizek and Ostadalova 1967, Ganther et al. 1972), the selenium was given as selenite. Therefore, in a first Se/Hg experiment, sodium selenite and methyl mercury chloride (MMC) were chosen as test chemicals.

However, in the root tips of Allium, neither the macroscopic nor the microscopic analysis pointed to any antagonistic effects of Se to MMC. Instead, a slight increase of damage effects was recorded for certain cytologic parameters.

Low concentrations of selenium not affecting the mitotic index, caused a slight increase in the frequency of chromosome aberrations. This was expressed as chromosome stickiness and weak c-mitotic effects (vagrant chromosomes, V, Fig. 2a), which would lead to imbalance in chromosome segregation (aneuploidy). Also by the combined Se/Hg treatments vagrant chromosomes may occur (V, Fig. 5e). In a workshop on systems to detect induction of aneuploidy, Önfelt and Ramel reported on such effects from organic mercury treatments (Zimmermann et al. 1979), and Levan

(1945) found that non-disjunction of anaphase chromosomes seemed to be a common characteristic caused by metal salts.

As will be shown later, results from Se/Hg experiments with V 79 cells (see Table 3, below) were not in accordance with the results in the Allium test (above). However, since the time for Se absorption into the cells has not been taken into consideration here, the discrepancy in results may be partly explained by the pretreatment with selenite of the V 79 cells (3 h prior to the Se/Hg co-treatment).

The enigmatic selenium

Selenium is an element of great versatility. Its toxicity is well known (e.g. Laveskog et al. 1976), and it has been reported to be carcinogenic in rats (Tscherkes et al. 1963) as well as mutagenic in bacteria (Löfroth and Ames 1978, Noda et al. 1979).

On the other side, many reports tell the opposite: Se is an element essential to many organisms, being incorporated in proteins, of which up till now only one, glutathione peroxidase, is known as a selenium dependent enzyme (Stadtman 1980). Se is also counteracting the toxic effect of certain heavy metal compounds (e.g. mercury) under experimental conditions (Parizek and Ostadalova 1967, Ganther et al. 1972) as well as under natural circumstances (Koeman et al. 1973, Ohi et al 1976).

Elevated levels of Se as well as of Hg were found in the organs of man and cats who died from the Minimata disease (Ohi et al. 1975). However, since Nishigaki and Harada (1975) found no increased Se parallel with elevated Hg levels in human umbilical cord and hair, there seems to be a discrepancy between the relative levels of Se and Hg in different human tissues.

The variability of selenium in biological effect is, as for most other chemicals, a question of dose; also the form in which it is active is of great importance. In a series of publications, Schwartz and Fredga (e.g. 1975) have demonstrated the variability in the biological potency of organic selenium compounds. Of five inorganic Se compounds with different Se valence states all but

selenate induced sister-chromatid exchanges in human blood cultures (Ray and Altenburg 1980).

Concerning the diets of mammals, naturally occurring organic Se is reported to be more effective than the inorganic selenite (Ku et al. 1972). However, for the counteraction of diseases in humans caused by lack of Se, sodium selenite is used and also Se-methionin (Ahlrot-Westerlund 1980).

Anti-carcinogenic effects of Se have been shortly reviewed by Ahlrot-Westerlund (1980). That Se has anti-carcinogenic effects indicates that an anti-mutagenic effect should be possible to observe in common screening tests.

Se/Hg interactions in the V 79 test

In a series of experiments with inorganic and organic selenium compounds, these were tested against certain mercury compounds in a short-term test using mammalian cells in culture (the Chinese hamster cell line V 79-4).

In the first experiment with inorganic Se and Hg both toxicity and mutagenicity parameters were taken into consideration. However, by the following tests on several Se and Hg compounds, the experiments were restricted to the less extensive toxicity/antitoxicity tests.

The following preliminary results not only elucidate possible interaction patterns between Se and Hg but also serve as an example of the complexity of these interactions.

Treatment with selenite/mercuries: A planned series of Se/Hg experiments was started with treatments of the V 79 cells by the inorganic compounds sodium selenite and mercury chloride. Both compounds are water soluble. Selenite was added to the medium some 10 minutes before addition of the mercury chloride (three concentrations) and a 3 h co-treatment was performed.

In this experiment antitoxic as well as antimutagenic effects were observed (Table 3). The concentration of Se was chosen to be non-toxic by itself, though Se in this concentration showed to be slightly mutagenic. The concentrations of Hg were chosen

near to the threshold value for toxicity (around 1 $\mu\text{g/ml}$ for HgCl_2), and toxicity was measured as percent of survival of seeded cells. At least in the two lower concentrations of Hg the toxicity was completely counteracted by the co-treatment with selenite. Both aza^R and oua^R mutants were recorded. When Se was present during the Hg-treatment, the frequency of both mutants was remarkably lowered compared to the experiments without Se.

Obviously Se and Hg are interacting in some way, but, considering that Se was added a few minutes before Hg, Hg may have become inactivated by Se before entering the cells.

To be sure that Se had been already incorporated in the cells before the co-treatment, another toxicity experiment was performed after pretreating with selenite for 3 h. The co-treatments here involved three different mercury compounds (HgCl_2 , MMC, MOEMC). As in the previous experiment, the results showed an obvious decrease of the toxic effects of mercury after co-treatment with Se. Thus selenite seems to react in an antagonistic way to all the mercuries tested.

Table 3. Toxic and mutagenic effects on V 79 cells after treatment with mercury chloride (HgCl_2) with or without previous addition of Se (as sodium selenite, $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$, 5 $\mu\text{g/ml}$). The controls are: One with Se, one positive control with N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and one negative control (medium). The results are means of 8 replicates $\pm$ S.E.

Treatment		Survival in %	
$\times 10^{-6}\text{M}$	$\mu\text{g/ml}$	Without Se	With Se
Hg			
14.8	4.0	59.0 $\pm$ 2.3	91.6 $\pm$ 3.8
7.4	2.0	84.0 $\pm$ 3.2	106.6 $\pm$ 2.8
6.0	1.5	93.7 $\pm$ 4.2	105.1 $\pm$ 3.4
Se			
63.0	5.0	-	97.9 $\pm$ 1.2
MNNG	0.5	18.1 $\pm$ 2.2	NT ¹⁾
Control		103.1 $\pm$ 1.4	-

		aza ^R mutants/ 10^4 survivors	
		Without Se	With Se
Hg			
14.8	4.0	28.4 $\pm$ 1.9	11.5 $\pm$ 1.3
7.4	2.0	19.3 $\pm$ 1.3	11.0 $\pm$ 1.7
6.0	1.5	13.6 $\pm$ 1.7	10.8 $\pm$ 1.0
Se			
63.0	5.0	-	8.1 $\pm$ 0.6
MNNG	0.5	298.1 $\pm$ 30.8	NT
Control		1.5 $\pm$ 0.4	-

		oua ^R mutants/ 10^4 survivors	
		Without Se	With Se
Hg			
14.8	4.0	2.8 $\pm$ 0.5	0.7 $\pm$ 0.3
7.4	2.0	1.7 $\pm$ 0.4	0.8 $\pm$ 0.2
6.0	1.5	1.5 $\pm$ 0.4	0.6 $\pm$ 0.2
Se			
63.0	5.0	-	0.6 $\pm$ 0.6
MNNG	0.5	308.6 $\pm$ 10.9	NT
Control		0	-

1) NT = not tested

The next step was to investigate if the Se pretreatment was capable of a counteracting effect in a subsequent Hg treatment. After pretreatment with selenite (1 $\mu\text{g/ml}$ for 20 h or 5 $\mu\text{g/ml}$ for 3 h), all the selenium-containing medium was removed and the mercury was added in new medium (Table 4). No antagonistic effects were noticed in this experiment; on the contrary the pretreatment of Se seemed to cause an additional toxic effect.

Table 4. Toxicity test on V 79 cells by three different mercury treatments and two different Se pretreatments (sodium selenite; 1 $\mu\text{g/ml}$ for 20 h or 5 $\mu\text{g/ml}$ for 3 h). The selenite was here removed prior to mercury treatment. Results from 6 replicates $\pm$ S.E.

Treatment			Survival in %		
			Without	After Se-pretreatments	
$\times 10^{-6}\text{M}$		$\mu\text{g/ml}$	Se	1 $\mu\text{g/ml}$	5 $\mu\text{g/ml}$
HgCl ₂	3.7	1.0	41.5 $\pm$ 5.8	43.9 $\pm$ 12.6	31.7 $\pm$ 11.4
MMC	0.3	0.1	73.0 $\pm$ 16.3	58.5 $\pm$ 7.4	40.9 $\pm$ 6.7
MOEMC	0.3	0.1	76.2 $\pm$ 9.4	67.5 $\pm$ 8.1	32.0 $\pm$ 2.6
MNNG		0.5	10.0 $\pm$ 2.1	4.0 $\pm$ 1.3	6.0 $\pm$ 1.9
Control			83.3 $\pm$ 10.0	79.6 $\pm$ 12.5	67.8 $\pm$ 8.5

Table 4 also shows the relative toxicity of the three mercury compounds, with MOEMC as the most toxic and the inorganicHg⁺⁺ ions being by far the least toxic. This is in accordance with a characteristic shared by all heavy metals (Clarkson 1977); the conversion of a metal cation to an organo-metallic species usually results in a dramatic change in the biological properties of the metal.

Treatments with organic Se-compounds/mercuries: Preliminary studies on V 79 cells and also other reports (Nakamuro et al. 1976, Ahlrot-Westerlund 1980) indicate that different Se-compounds have different biologic activity.

The hitherto latest experiment with Se was therefore designed to investigate eventual co-reactions between each of three mercuries (HgCl_2 , MMC and MOEMC) and two organic selenium compounds (selenomethionine and seleno-cystine).

The following treatments were performed: Pure medium (control), one concentration of each of the five chemicals, and combinations of each of the selenium compounds and each of the mercury compounds - partly in the form of a "co-treatment" (simultaneous treatment with Se and Hg), and partly as "separated treatments" (pretreatment with Se 20 h followed by Hg treatment, 3 h, in a Se-free medium). For comparison, the medium was also changed in the experiments with no pretreatments (Table 5). Toxic effects are measured as number and size of surviving colonies after various treatments.

Table 5. Toxic effects on V 79 cells after various treatments or co-treatments with Se and Hg compounds. 200 cells were seeded in each petri dish; 3 petri dishes for each treatment. The mean values are given $\pm$ S.E.

Treatment	No. of surviving colonies after treatment or co-treatment	No. of surviving colonies after medium change or separated treatments
Control (medium)	160.6 $\pm$ 6.1 small big	163.3 $\pm$ 17.6 small big
Se-methionine 1 $\mu\text{g/ml}$	150.3 $\pm$ 12.9 small	142.6 $\pm$ 8.3 small
Se-cystine 1 $\mu\text{g/ml}$	158.3 $\pm$ 9.8 big	178.6 $\pm$ 16.4 big
HgCl_2 2 $\mu\text{g/ml}$	114.0 $\pm$ 8.5 small	118.0 $\pm$ 10.0 small
MOEMC 0.1 $\mu\text{g/ml}$	51.6 $\pm$ 3.9 small	46.3 $\pm$ 3.4 small
MMC 0.2 $\mu\text{g/ml}$	171.0 $\pm$ 12.1 small	164.6 $\pm$ 15.2 small
Se-meth/ HgCl_2	117.6 $\pm$ 21.2 small	167.3 $\pm$ 12.3 small
Se-meth/MOEMC	65.3 $\pm$ 12.7 small	106.6 $\pm$ 2.3 small
Se-meth/MMC	130.0 $\pm$ 11.5 small	167.3 $\pm$ 6.4 small
Se-cyst/ HgCl_2	170.0 $\pm$ 20.6 big	197.0 $\pm$ 7.5 big
Se-cyst/MOEMC	190.0 $\pm$ 21.0 big	195.0 $\pm$ 7.5 big
Se-cyst/MMC	187.6 $\pm$ 29.1 big	193.3 $\pm$ 6.9 big

From the results given in Table 5 the following conclusions may be drawn: The mercury compounds are all exerting a certain toxic effect on cell growth. (The low concentrations of mercury were intentionally chosen to ensure a certain amount of survival even in the case of additional effects after the co-treatments.)

The Se compounds alone acted in slightly different ways: Se-methionine being weakly toxic (only small colonies) and Se-cystine instead giving a slight positive growth effect (only big colonies).

When Se-methionine (Se-meth) was administrated together with one of the mercuries, there were neither antagonistic nor additive effects except for Se-meth/MMC where the Se-meth additionally lowered the number of surviving colonies. The removal of Se-methionine before the Hg treatment improved the results (more surviving colonies) for HgCl₂ and MOEMC, and only a little for MMC. This means that Se-methionine, though not giving much effect by itself, to some extent may prepare the cells to counteract the toxic effect of the mercuries.

Concerning the Se-cystine (Se-cyst), there is a clear improvement of growth conditions; there are more and bigger colonies both with and without a continued presence of the pretreating agent. This may mean that Se-cystine has easily passed into the cells and that this compound has the capability of counteracting the mercuries. In addition the Se-cystine seems to improve the cell growth to a better level than in the control medium.

Compared to the effects of the inorganic selenite, where there was no counteracting effect to Hg when the selenite was removed prior to Hg treatment, the Se-cystine pretreatments have resulted in a cellular state of defence against mercury.

Thus, of the three Se compounds tested, Se-cystine is the compound most actively interfering with the Hg metabolism in the V 79 cells.

Selenium forms the active site of the enzyme glutathion peroxidase (Stadtman 1974), and this enzyme can be inhibited, in vitro as well as in vivo, by heavy metals (Ahlrot-Westerlund 1980). Selenium also seems to protect the microsomal function by induction of glutathion peroxidases (Olsson 1981). Since V 79 cells do not contain the microsomal MFO-system (Huberman and Sachs 1874), it is possible that other results may be achieved by a cell-mediated test (with MFO-metabolism) on Se/Hg interactions. Actually, Lo et al. (1978) found, in experiments with human fibroblasts, that selenite (but not selenate) could be "activated" by liver microsomes and cause increased effects (chromosome aberrations, DNA-repair synthesis and a lethal effect).

The above mentioned results on the mercury/selenium interactions in the V 79 cell system, and also earlier results on Allium (V), demonstrate the different patterns encountered in plant and mammalian cells, also following the use of inorganic and organic Hg and Se compounds in the form of co-treatments or subsequent treatments.

To fully understand the meaning of these results, continued experimental efforts, e.g. mutagenicity tests and tests with metabolic activation, are needed. The available data may be useful for the planning of new experiments for further elucidation on these complex interactions.

CHROMOSOME BANDING IN ALLIUM (VI, VII, VIII)

As a part on the studies on the applications of various test methods it is important to increase the knowledge of the test material used. Of the four test methods applied in this study, the Allium test has been most frequently used and even further developed (V). By C-banding of the chromosomes in Allium cepa, an attempt to improve the possibilities of getting information from chromosome analysis has been performed as well (VI). Similar results by C-banding of A. cepa have been obtained later (e.g. Stack 1974, Tse-li et al. 1978).

By application of the C-banding modification depicted in VI, on root tip material from three different Allium species, an earlier hypothesis of relationship between the three species (Levan 1940) could be confirmed (VII). Within the C-banded genomes, 3 or 4 chromosome pairs could be identified by centromeric, telomeric or intercalary bands. Among the three species, the C-bands of A. fistulosum were the best stained and most easy to recognize, which makes this species a suitable material to study the effects of chromosome breaking agents. However, for a large scale screening test, small onions of A. cepa are the material most easily available.

A main purpose of the study of chromosome banding in Allium was that a differentiated chromosome pattern may be helpful in determining more exactly where in the chromosome complement an eventual specific action of an agent is manifested. However, when the two carcinogens benzo(a)pyrene (BP) and N-methyl-N-nitro-N-nitrosoguanidin (MNNG) (VIII) were used as test chemicals, and also when certain organic mercury compounds were used (Fiskešjö, unpublished), the subsequent C-banding failed and instead the whole cytogenetic appearance was seriously deteriorated. Thus, chromosome banding procedures seem to be too sensitive to any "pretreatment" by other chemicals.

Instead other "banding methods" for Allium chromosomes may be revealed when studying the effects of metal ions in the Allium test. Promising results point to certain banding effects following the use of beryllium ions (Fiskesjö 1982a).

ALLIUM ROOT TIP CELLS POSSESS THE MFO-SYSTEM

The problems of testing materials with different metabolic pathways are dealt with in VIII, where the results in the Allium test are compared to those in the V 79 test reported elsewhere (e.g. Huberman and Sachs 1974, Fiskesjö 1982b). The V 79 cells do not possess the mixed-function oxidase(MFO)-system necessary for metabolizing polycyclic hydrocarbons, e.g. benzo(a)pyrene (BP), which have to be metabolically activated before exerting their biological effects. Other chemicals, as N-methyl-N-nitro-N-nitrosoguanidin (MNNG), induce effects even without the MFO-system.

MNNG gave similar results in Allium as in the V 79 cells not supplemented with the MFO-system. Compared to the positive effects with V 79 cells in a cell-mediated test (addition of the MFO-system), BP gave clearly lesser effects in the Allium test. However, in spite of certain difficulties to keep BP in a solved state in the water solutions necessary for the performance of the Allium test, still there were clear damage effects as restrictions of root growth as well as chromosomal damage and disturbances in cell division.

These results indicate that Allium cells possess the important MFO-system and that tests with both Allium and V 79 cells (in the cell-mediated test) give a response in the same direction even for a group of chemicals (polycyclic hydrocarbons) which have to be metabolically activated before causing biological effects.

CONCLUDING REMARKS

The correlation between results

The toxic effects of two mercuries was shown to be in very good accordance in the four test systems used (Table 2).

On the basis of the wide chemical knowledge on mercury compounds, also considering the fact that these compounds do not need any help (e.g. the MFO-system) in metabolism, this agreement in effect could be expected. That this similarity could be shown in 4 different short-term test systems actually serves to indicate the reliability of the test systems.

Concerning selenium, it may be added that the differences in effects from different Se compounds in the various test systems may depend on the relatively short time of treatment. Richold et al. (1977) found that there were differences in the initial utilization of the labelled Se compounds selenite and seleno-methionine in vivo, but after a week, Se from both sources appeared to be metabolized similarly. As was shown earlier, the results in the present investigation pointed to a certain variation between the effects of the different Se compounds. It is possible that such discrepancies in results in short-term tests in vitro compared to results in vivo may be diminished when Se is administrated during a longer period of time. The results from the Se/Hg co-treatments in short-term tests may, in a continued research program, contribute to the understanding of the action of these chemicals in vivo.

However, it is a common statement that high correlations exist between the occurrence of chromosome breaks and mutagenicity and between mutagenicity and cancer, and also between different short-term test systems (e.g. Hollstein et al. 1979). Thus, the above cited weak tendency of mercury to cause chromosome breaks and also a low incidence of mutagenicity, should support the assumption that mercury compounds to a certain low degree may contribute to the development of human cancer.

The small doses are the most dangerous

High doses of a genotoxic chemical most often cause obvious damage effects which may lead us to avoid that chemical.

From a genetical point of view, however, the small doses are a greater threat to the human population and to natural ecological systems since they lead to a higher mutation rate based on small changes in the inheritable material which allow survival of the afflicted individuals.

It is stated that an artificially increased mutation rate is potentially capable of producing a general decline in genetic health (Committee 17, 1975), and it is supposed to be a logical conclusion that an abrupt significant increase in the mutation frequency of any species, including man, is a serious threat to the future of the species (Hemminki et al. 1979).

The use of short-term test systems is an attempt to get information on the action of chemicals with the main purpose of avoiding those which are mutagenic/carcinogenic. There has been a wide discussion if there exist threshold values or not (Maugh 1978a), and also to what degree complex metabolism routes in vivo decrease the danger from many carcinogens.

On the other hand, some chemicals may be latent mutagens in very low doses and later on, promoted by other agents, change to active mutagens. This two-step model of carcinogenesis (Berenblum 1941, 1969), is now accepted and demonstrated by the use of short-term test systems (Lankas et al. 1977, Huberman et al. 1979). These aspects, including increased risks by long-time exposure, make it quite clear that positive effects of low doses in a short-term test system should be seriously considered.

A mathematical model for calculation of the total risk for humans was presented by Hussain and Ehrenberg (1977). The extrapolation of man was based upon calculations including for instance metabolism and time of exposure.

When the dose is small, and the dose response of mutagenicity falls within a very narrow interval of concentrations tested (see for example Fig. 2 in IV or Fig. 1 (below), showing the effects of 2,4-dichlorophenol, there may be a risk for overlooking the

mutagenicity. This may be expressed by the words of a modern author (Tikkanen 1980): "The big catastrophe is that we do not see it".

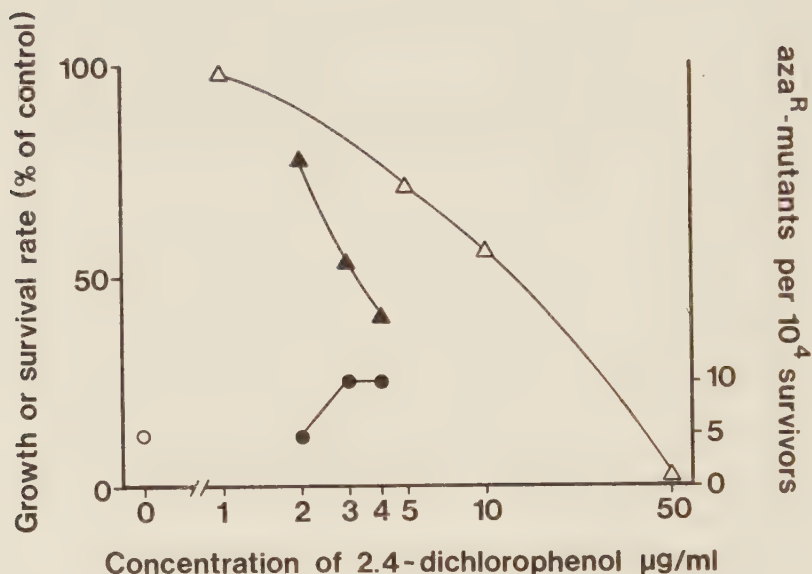


Fig. 1. Toxicity and mutagenicity of 2,4-dichlorophenol: Growth rate (measurements of Allium root length) (Δ — Δ) and survival of V 79 cells ($\blacktriangle$ — $\blacktriangle$), left ordinate; compared to the incidence of V 79 aza^R mutants after treatment ($\bullet$ — $\bullet$) and in control medium ($\circ$), right ordinate. Note that significantly increased amounts of mutants occur only within the narrow interval of treatments from 3 to 4 μ g/ml (no survival at 5 μ g/ml).
(Data from Fiskesjö et al. 1981 and Fiskesjö 1982b, in preparation).

The usefulness of the tests

"To reduce the probability of false negative and false positive results, it seems desirable to use a battery of tests rather than any test in isolation" (de Serres 1976a). That a battery of tests is the only reliable model for testing is commonly acknowledged (e.g. Committee 17, 1975, de Serres 1976b, Hollstein et al. 1979).

My contribution to this field of investigation is a comparison of effects from mercury compounds in 4 different test systems and the choice of two systems (V 79 and Allium) for further use. Both these test systems have been shown to be useful in a "battery screening" of environmental chemicals (e.g. Fiskesjö 1979b, 1980, 1981d) and also for basic research, as for instance on the effects of metal ions and their interactions (Fiskesjö 1979a, 1981b, 1982a).

The advantages and the relevance of the two selected tests are shortly reviewed below:

The V 79 test Epidemiological surveys provide evidence that some chemicals in our environment are responsible for a significant proportion of human cancers (Higginson 1972). The mechanism by which chemicals induce malignancy is not fully known. In view of the complexity of studying carcinogenesis in the whole animal, it is useful to employ simpler systems such as mammalian cells in vitro, which can be used to elucidate fundamental cellular effects of chemical carcinogens (Huberman and Langenbach 1978).

Furthermore, mammalian cells and especially the V 79 cell line, are pointed out as an important part of test batteries for the screening of environmental chemicals (e.g. Hollstein et al. 1979).

The V 79 cell line is convenient to use for mutagenicity studies since it has a rapid growth rate, high plating efficiency and a stable karyotype (Bradley et al. 1981). Moreover, the two gene markers most frequently used (HGPRT locus and $\text{Na}^+/\text{K}^+\text{ATPase}$ locus) are shared with many other mammalian species (for review see IV).

The V 79 system is applicable for direct acting chemicals and also, by co-cultivating with cells having the MFO-system, for chemicals which have to be metabolically activated (Huberman and Sachs 1974, 1976, Newbold et al. 1977).

The Allium test system The Allium test gives a clear information of toxic effects caused by a test chemical; toxicity is easily observed by growth restrictions or growth aberrations. By cytological studies information on genotoxic effects may be recorded as well.

There is a high correlation between chromosomal damage and mutagenic events: "No agents are known which cause chromosome breakage but not gene or point mutations" as pointed out by deSerres (1978), who also draws the conclusion that "Plant systems seem especially well suited for research in at least the areas of basic mechanisms, screening, and environmental monitoring".

Even other scientists (e.g. Grant 1978) give their prominence to plant systems as environmental monitors: "Higher plant systems appear to be excellent indicators of the cytotoxic, cytogenetic, and mutagenic effects of environmental chemicals" and "a number of studies which have been carried out on pesticides indicate that there is an excellent correlation between chromosome abnormalities found in root tip systems and those found in mammalian cell systems".

Concerning the usefulness of plant systems in tests of chemicals which have to be metabolically activated, Vig (1978) says: "The plant systems, in my opinion, have a place in at least the preliminary screening of new chemicals being poured out into the environment. The wonderful ability of plants to activate promutagens may be of great interest, particularly in view of the fact that all of us ultimately have to consume plant materials, many or perhaps all of which have been treated with a variety of agents. From this point alone the study of plant systems should be seriously considered".

The remarks cited above were all expressed by a workshop on "Higher plant systems as monitors of environmental mutagens" and show the wide interest in using plant test systems. The reasons are many: Plants are easy to handle, and there are most often good chromosome conditions, low cost and, most important, the above mentioned good correlation to other test systems.

Moreover, the Allium test is a suitable system for the detection of induced aneuploidy; a parameter proposed to be one of four types of effects caused by chemicals: 1. gene mutations, 2. chromosomal damage, 3. non-disjunction, 4. primary DNA-damage (ICPEMC 1979). Agents which cause aneuploidy are often neglected. However, Hsu (1976) emphasized the importance of investigating this type of reaction, and deSerres stated: "The most promising area for future development is a screening test for non-disjunction. Development of such an assay is absolutely essential for a comprehensive screen, since chemicals exist which can cause non-disjunction but no other classes of genetic alterations". In a review on the use of mitotic chromosomes as a method for the detection of possible genetic damage, Brögger (1979) has introduced the term turbagenic effects for this type of mitotic disturbances.

In the search for relevant short-term test systems the Allium material is listed among other systems under the headings "Promising bioassays for the detection of chemical carcinogens" (Stich et al. 1975).

Thus, positive results in the Allium test should be considered as a warning and also an indication that the tested chemical may be a risk to human health and to our environment.

SUMMARY

The use of certain short term tests for environmental chemicals was evaluated by studying the effects of mainly two mercury compounds (methyl mercury chloride, MMC; methoxyethyl mercury chloride, MOEMC) in 4 different test systems: Allium root tips, human lymphocytes, lysogenic bacteria and mammalian tissue cultured cells (V 79 cell line). The counteracting effect of Se to Hg was investigated in the Allium and V 79 test systems. For a more specific evaluation of the Allium test, the effects of two carcinogens (benzo(a)pyrene, BP and N-methyl-N-nitro-N-nitrosoguanidine, MNNG) were tested in this system. Also a modified method for chromosome banding in Allium was described.

The original Allium test has been modified, e.g. by using series of test onions. The parameters studied were: root growth and the usual cytological criteria such as the mitotic index, c-mitosis and chromosome aberrations. By the study of lymphocytes in vitro, mainly c-mitosis were recorded. In the experiments with lysogenic bacteria E. coli K39(λ) was used. The test with mammalian cells (Chinese hamster cell line V 79) comprises studies on toxicity (survival) as well as mutagenicity (selection of mutants resistant to 8-azaguanin or ouabain).

Tests with MMC and MOEMC gave similar results in all the 4 systems used: the toxicity level ranged from 0.8 to $2.0 \times 10^{-6} \text{M}$ (MMC) and from 0.3 to $1.0 \times 10^{-6} \text{M}$ (MOEMC). Mutagenicity, however, was observed in doses below the lowest level of toxicity. Thus there is a risk for overlooking mutagenic effects. Antagonistic effects of selenite to inorganic mercury could not be observed in the Allium test, but, of three selenium compounds tested, Se-cystine seems to be antagonistic to Hg in V 79 cells. Weak c-mitotic effects (vagrant chromosomes), which may lead to

aneuploidy, were observed in Allium root tip cells after Se and Se/Hg treatments. The chromosome banding in Allium revealed 3-4 identifiable C-banded chromosome pairs of the 8 pairs in A. cepa and A. fistulosum. Application of banding in combination with certain test chemicals (Hg, BP, MNNG) caused a deterioration of the quality of the C-bands. Therefore the ordinary orcein squash method for cytological preparations was preferred for rapid screening. A comparison of the effects of BP and MNNG showed that the directly acting MNNG gave similar results in Allium as in V 79 cells, while BP, which has to be metabolically activated by cellular enzymes (the mixed-function oxidase(MFO)-system) gave different effects in the two test materials. In the V 79 cell line, which is lacking the MFO-system, effects of BP were obtained only when the MFO-system was introduced into the system. In Allium, however, clear effects of BP could be recorded without any additional metabolism, which showed that the Allium material itself possesses the MFO-system. This emphasizes the wide applicability of the Allium test. Especially the Allium and the V 79 tests are proposed as a part of a test battery, necessary for reliable testing of chemicals.

The effects of low doses should be recorded in short term test systems in order to avoid the misuse of mutagenic/carcinogenic compounds which otherwise would affect the genetic material in the human population and the environment.

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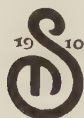
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GEIRID FISKESJÖ

SOME RESULTS FROM ALLIUM TESTS
WITH ORGANIC MERCURY
HALOGENIDES

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SOME RESULTS FROM ALLIUM TESTS WITH ORGANIC MERCURY HALOGENIDES

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MATERIALS AND METHODS

AS part of a planned study on the influence of biocides in various cellular systems—microbial, botanical and zoological—the present paper will report on some results obtained with one commercial fungicide, in which the active ingredient is an organic mercury compound, and with four chemically defined related mercurials. The former preparation, betoxin, was obtained from Ewos Co. and was declared by the manufacturers to consist to 90 % of “ethyl mercury halogenide”. The latter substances were three chlorides, methyl, ethyl and methoxyethyl chloride and one bromide, butyl mercury bromide. The latter compound, which at an earlier investigation at our institute (FAHMY, 1951) had shown especially interesting effects, was kindly provided by Prof. ARNE FREDGA, Uppsala. In the continuation the four chemically defined compounds will be referred to by the following abbreviations:

methyl mercury chloride	MMC
ethyl mercury chloride	EMC
methoxyethyl mercury chloride	MOEMC
butyl mercury bromide	BMB

There was some difficulty to bring the substances into solution. Betoxin, a red powder, was utilized in the form of water suspensions prepared from different amounts of the substance corresponding to 0.02 to 6 % (by weight). The three chlorides were dissolved in water by shaking and warming but not boiling on waterbath. The BMB was not dissolved completely by this treatment, but after 24 hours of alternate warming and shaking only a very small amount remained undissolved and was disregarded at the preparation of the lower con-

centrations. The concentration ranges tested may be seen from Table 2.

The *Allium* roots were grown in tap water at room temperature to an average length of 2—3 cm and then transferred to the different solutions to be tested. Each experimental series included one or more control bulbs. Three standard treatments were used: 4 hours, 24 hours and 24 hours followed by a 24 hour period of recovery in tap water. In the tables these treatment times are designated 4, 24 and 24+24, respectively. Root tips were fixed in a mixture of 9 parts 45 % acetic acid and 1 part 1 N hydrochloric acid, macerated in the fixative for five minutes at 60° C and squashed in 2 % orcein in 45 % acetic acid.

All substances except BMB were tested in at least two experimental series. To avoid possible sources of error, freshly prepared solutions were always used, and onions were selected of about the same size, 2—3 cm in diameter. Whenever possible, c-mitosis was estimated in samples of 50 metaphases and anaphases in each treatment.

GENERAL OBSERVATIONS

As expected, the highest concentrations acted immediately killing on the cells and in many cases the chromosomes became more or less fixed by the treatment. Thus, in the highest concentrations, normal mitoses are often found as a result of the fixation of the cells. In these cases, the chromosomes often exhibit a highly sticky appearance. Interesting chromosome structures were often revealed in these high concentrations. Thus, heterochromatic regions appear, and the chromosomes may show a mottled structure of darker and lighter areas. Often the centromeric and telomeric regions take stain more deeply (positive heteropycnosis, Fig. 1). Also the chromatids often appear subdivided, half-chromatids being visible and forming a relational spiral of the second order (Fig. 1 b, c). Towards lower concentrations the fixing activity subsides, toxicity often effects the disappearance of mitosis, and after longer treatments there is usually a concentration zone without mitoses. At still lower concentrations, c-mitosis becomes the typical feature, often with an incidence approaching 100 %. This concentration zone passes into the still lower zone where mitoses are normal.

The threshold of c-mitosis, i.e. the step from the lowest concentration giving undisputable c-mitosis to the highest concentration with normal spindle action, is often characterized by partial inactivation of the spindle. In this zone, which in the present treatments can be rather wide, all kinds of transitions are found between c-mitosis and normal

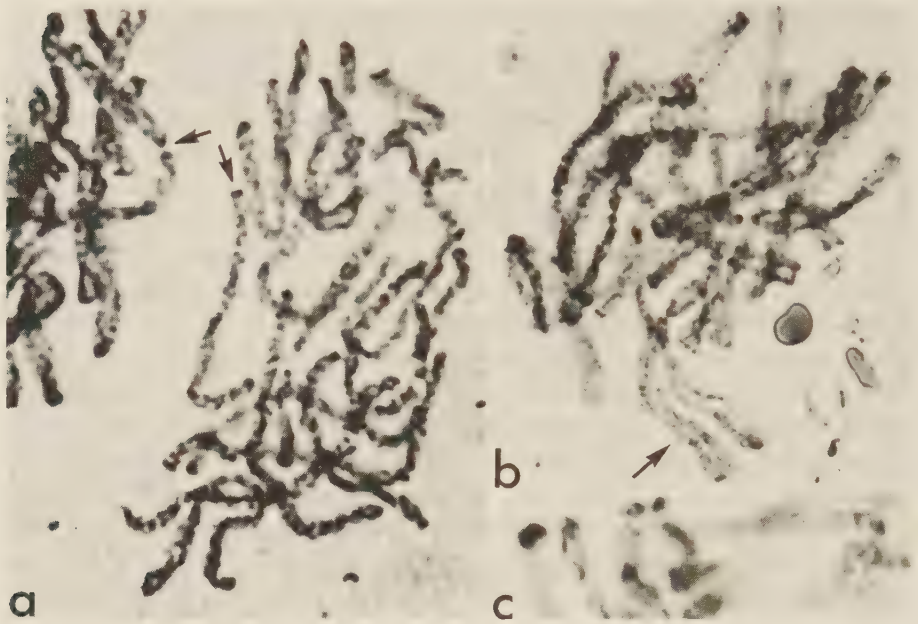


Fig. 1. Root chromosomes of *Allium cepa* exposed for 4 hours to $100 \times 10^{-6} \text{M}$ of ethyl mercury chloride; strong toxic action with the appearance of heterochromatic differentiations; a, left arrow: centromeric heterochromatin, right arrow: telomeric heterochromatin; b and c: subdivision of the metaphase chromatids, even $1/2$ chromatids may be seen. — a and b: $\times 1600$; c: $\times 2300$.

mitosis, such as multipolar and monopolar mitoses. In this concentration range also chromosome fragments and bridges were seen, showing that chromosome breakage was induced by the present treatments. Since the incidence of clear chromosome breaks was rather rare, no attempts were made to determine their exact frequencies, but they were undoubtedly more frequent in the treatments than in the controls, where breaks were seen very rarely. The c-tumor reaction was found in all treatments, usually in one or more of the weakest concentrations giving c-mitosis.

SPECIFIC OBSERVATIONS IN THE DIFFERENT COMPOUNDS

The results with betoxin are presented in Table 1. Toxicity, as judged from loss of turgor of the roots, reached down to 0.2 % after 24 hours. The c-mitotic threshold was between 0.2 and 0.02 %, thus far below the concentrations recommended in agricultural practice (2—6 % for

TABLE 1. *Betoxin*.

Concentration weight	Toxicity loss of turgescence		Mitoses		C-mitoses		Normal anaphases		Sticky chromosomes	
	4	24	4	24	4	24	4	24	4	24
%	4	24	4	24	4	24	4	24	4	24
6	—	+	++	±	—	—	+	—	+	+
3	—	+	+	±	±	—	±	+	+	+
2	—	±	+	±	+	—	±	—	+	+
0.6	—	±	+	+	+	++	—	—	—	—
0.2	—	±	+	—	++	—	—	—	—	—
0.02	—	—	+	—	—	—	+	+	—	—
control	—	—	++	++	—	—	++	++	—	—

different kinds of seed). Chromosome breakage was observed occasionally in higher concentrations. It may be mentioned in passing that two other biocides from Ewos Co. were tested, viz. rotoxol (DDT and lindan) and a nicotine preparation, both of which induced c-mitosis and chromosome breakage.

The results from the other substances, MMC, EMC, MOEMC and BMB, are surveyed in Table 2, in which the upper half records macroscopic observations, loss of turgor from the roots and presence of c-tumors or other growth deviations, and the lower half microscopic observations, percentage of cells with c-mitosis and occurrence of mitotic inhibition.

Loss of turgescence is a reliable sign of toxicity having caused irreversible damage. On the other hand, presence of c-tumors is a sign of enough viability to permit certain growth processes to go on. The four compounds studied show roughly similar toxicity levels with some indication that the toxicity threshold is falling (=toxicity increasing) in the order MMC, EMC, MOEMC and BMB (Table 3).

In the three alkyl halogenides, the c-tumor reaction was of the ordinary type with the elongation zone of the root developing a swelling. In the MOEMC, a characteristic variant of the c-tumor reaction was found, known from some earlier experiments (*e.g.* LEVAN, 1945) and called the "crochet-hook" effect by EHRENBURG *et al.* (1949). This effect repeated itself in several tests in the same concentrations, viz. $1-2 \times 10^{-6}M$ and is evidently characteristic of this treatment. After a treatment of 24 hours, the 3—4 terminal mm of the root tips bent upwards making each root resemble a hook (Fig. 2 c). After longer time in the solution, the bending continued for one or more complete turns, making

TABLE 2. Toxicity, c-tumors and c-mitoses induced by four organic mercury compounds.

Reaction studied	Concentration $\times 10^{-6}M$	Methyl mercury chloride hours			Ethyl mercury chloride hours			Methoxyethyl mercury chloride hours			Butyl mercury bromide hours		
		4	24	24 +24	4	24	24 +24	4	24	24 +24	44	24	24 +24
Toxicity (loss of turgescence) c-tumors	2000	+	+	+	+	+	+						
	1000	+	+	+	+	+	+						
	500	$\pm$	+	+	+	+	+						
	200	-	+	+	+	+	+	$\pm$	+	+			
	100	-	+	+	-	+	+	-	$\pm$	+	-	+	+
	50	-	+	+	-	+	+	-	$\pm$	$\pm$	-	+	+
	20	-	$\pm$	$\pm$	-	+	+	-	-	$\pm$	-	+	+
	10	-	$\pm$	$\pm$	-	$\pm$	$\pm$	-	-	-	-	-	-
	5	-	$\ominus$	$\ominus$	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	$\ominus$	$\ominus$	-	$\square$	$\square$	-	-	$\ominus$
	1	-	-	-	-	-	-	-	$\square$	$\square$	-	$\ominus$	$\ominus$
	0.5	-	-	-	-	-	-	-	-	-	-	-	-
	0.2	-	-	-	-	-	-	-	-	-	-	-	-
	0.1	-	-	-	-	-	-	-	-	-	-	-	-
	0.05	-	-	-	-	-	-	-	-	-	-	-	-
	control	-	-	-	-	-	-	-	-	-	-	-	-
c-mitoses %	2000	0	0	0	0	²	0 ¹						
	1000	0	0	0 ¹	0	0	0 ¹						
	500	0	10	4 ¹	14	22	0						
	200	34	40	4	64	16	0 ¹	4	8	0			
	100	58	44	48	90	20 ¹	0	4	6	0	100	16	²
	50	84	42	21 ¹	71	24	0 ¹	2	2	0 ¹	90	40	100 ¹
	20	90	50 ¹	67 ¹	66	20	59	64	0	²	90	54	²
	10	90	²	96	100	0 ¹	²	90	0 ¹	²	90	²	²
	5	76	38 ¹	36	100	²	84	18	32	8	96	²	²
	2	4	6 ¹	0	34	94 ¹	91 ¹	8	8	4	100	²	100
	1	0	8 ¹	0	6	0 ¹	16	0	2	4	54	90	100
	0.5	0	0 ¹	0	10	14	²	2	2	4	42	47 ¹	28
	0.2	0	4 ¹	0	4	4 ¹	8	2	6	12	40	12	0
	0.1										16	0 ¹	3 ¹
	0.05										20	13 ¹	²
	control	0	0	0	2	0	0	0	2	0	2	0 ¹	0

+ roots slack; $\pm$ roots slackening; - roots turgescence; $\ominus$ c-tumors;
 $\square$ crocket hooks; ¹ less than 50 cells examined; ² no mitoses.

TABLE 3. *Threshold levels for toxicity, c-tumors and c-mitoses in four organic mercury compounds.*

Reaction studied	Time of treatment	Methyl mercury chloride	Ethyl mercury chloride	Methoxy-ethyl mercury chloride	Butyl mercury bromide
Highest concentration with no macroscopic toxicity	4	200	100	100	(100)
	24	5	5	20	10
	24+24	5	5	10	10
Concentrations showing growth disturbances	4	—	—	—	—
	24	5	2	1—2	1
	24+24	5	2	1—2	1—2
Lowest concentration with at least 50 % c-mitosis	4	5	5	10	1
	24	20	2	—	1
	24+24	10	2	—	1

the roots from irregular spirals (Fig. 2 a). If the roots were placed on tap water after 24 hours of treatment, the tips started growing perpendicularly in their normal way and the original bend remained as a knee at about the same level in all roots. The concentrations inducing c-tumors and other growth disturbances are recorded in Table 3.

It is seen in Table 2, and in the diagram of Figure 3, that all four substances have c-mitotic activity. In MMC, EMC and especially in BMB there are wide concentration ranges with high incidence of c-mitosis. In the MOEMC there are only two concentrations, and only at the first time of fixation, in which the c-mitotic incidence reaches 50 % or more. Both above and below the active c-mitotic zones, there are concentrations with normal bipolar mitoses. As pointed out above under 2, the normal mitoses above the c-mitotic zones are due to the fixation effect of the substances. Below the c-mitotic range there are usually one or more concentrations with a mixture of c-mitoses and normal mitoses, and here all kinds of transitions occur between complete spindle inactivation and normal spindle function. In the c-mitotic reaction, it is still more evident than in the toxic reaction that the threshold values form a falling series from MMC over EMC to BMB (Table 3), thus the c-mitotic activity shows correlation to increasing length of the alkyl chain of the molecule.

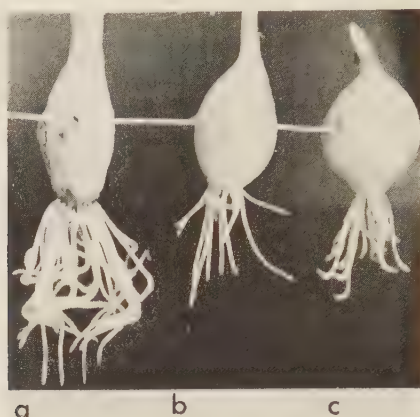


Fig. 2. The crochet-hook reaction induced by methoxyethyl mercury chloride at a concentration of $10^{-6}M$, a: 72 hours' treatment, b: control, c: 24 hours' treatment.

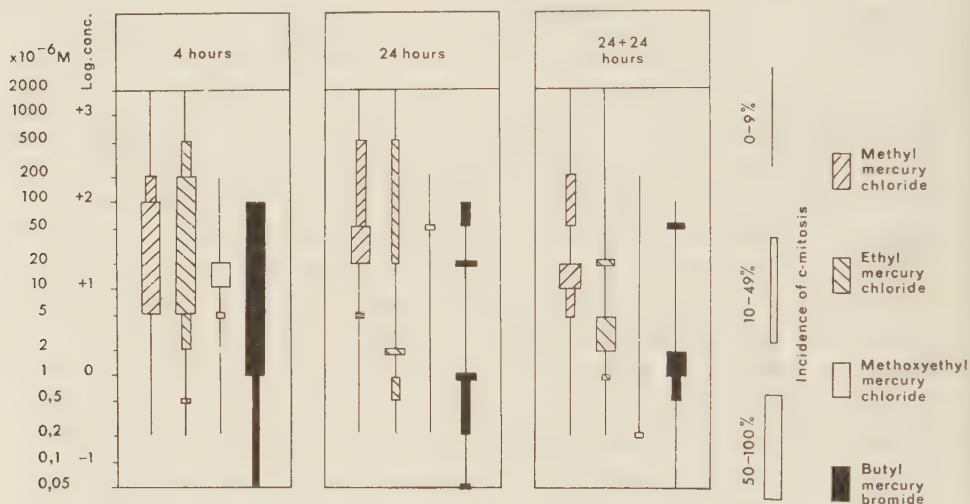


Fig. 3. Diagrammatic survey of the incidence of c-mitosis in the present experiments.

COMMENTS

The observations on toxicity, c-tumor and c-mitosis in the present investigation are in good accordance with the results of earlier workers in this area. Thus, FAHMY in his unpublished thesis (1951) made *Allium* tests with a great many organic mercury compounds including three of the present substances, viz. MMC, MOEMC and BMB, and RAMEL

(1968) made similar tests with among others MOEMC. In these studies the following threshold values for c-mitosis were given (the present values in parenthesis):

Compound	Author	Lowest concentration with c-mitosis $\times 10^{-6}M$		Highest concentration with normal mitosis $\times 10^{-6}M$	
MMC	FAHMY	1	(2)	0.5	(1)
MOEMC	FAHMY	5	(2)	2	(1)
	RAMEL	3.14		1.60	
BMB	FAHMY	0.1	(0.05)	0.05	—

It is interesting that the organic mercury compounds are so much more active as inducers of c-mitosis than colchicine, the threshold value of which is of the size order of 100 to $250 \times 10^{-6}M$. Because of their high toxicity, however, the mercury compounds are less efficient as inducers of chromosome doubling than colchicine, even though cases of accidental polyploidization have been known to occur after routine seed pretreatment with mercurial fungicides (LEVAN, 1951).

SUMMARY

Five organic mercury halogenides, including one technical preparation, betoxin, and four chemically defined substances, methyl, ethyl, methoxyethyl mercury chloride and butyl mercury bromide, were studied as to their effect in the *Allium* test. They all showed largely similar effects. With falling concentrations the following reactions were observed: fixation with induction of chromosomal stickiness and differential staining of heterochromatic regions, c-mitosis and c-tumors, various mitotic disturbances, as multipolar mitoses. In a low frequency also chromosome breakage was induced, noticeable as acentric fragments and anaphase bridges.

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GEIRID FISKESJÖ: The effect of two organic mercury compounds on human leukocytes in vitro

(Received November 3, 1969)

It has been demonstrated repeatedly that the effect on living cells of organic mercury compounds can be neutralized to a certain degree by the amino acid cysteine. In experiments with sea urchin eggs, RUNNSTRÖM and MANELLI (1964) found that a cysteine concentration 1000 times higher than that of the mercurial removed the effect of the latter even in cases when the cysteine was added after the mercury treatment. The active factor in the cysteine is the S-H groups, and it was possible to determine the amount of S-H groups in the eggs by varying concentration of the mercury compound. Also other workers (for reviews see BOYER 1959 and RAMEL 1969), have shown that mercury compounds react with S-H groups. Thus, RAMEL treated root meristems of onions with various mercury compounds and—simultaneously or after the treatment—added BAL (2,3-dimercaptopropanol) containing 2 S-H groups. In both cases, the effect of the mercurials, measured as incidence of c-mitosis, was counteracted. RAMEL's conclusion was that the antagonistic action of BAL depends on its capacity of combining with the mercurials and thus preventing them "from reacting with those S-H groups directly or indirectly involved in the spindle formation" (l.c., p. 226).

MIKAELSEN and PEDERSEN (1968) demon-

strated the protecting effect of cysteine against X-rays in seeds of *Allium cepa*. In their experiments seeds were treated with cysteine—HCl one hour prior to irradiation. The authors suggest that "the sulfhydryl compounds are scavenging primary or secondary radicals from the radiolysis of water and thus are involved in mechanisms where chromosome breaks are produced by indirect effects of radiation" (l.c., p. 404).

In experiments with human leukocytes, JACKSON and LINDAHL-KIESSLING (1963) demonstrated that another chemical compound with S-H groups, beta-mercaptopyruvate, may interfere with the pathways of cysteine, disturb the spindle mechanism and inhibit centriole duplication without inhibiting DNA synthesis. The significance of S-H groups in the mitotic apparatus was pointed out by MAZIA (1955). According to JACKSON and LINDAHL-KIESSLING, human cells contain cysteine, but in large amounts only in tissues such as skin and hair follicles.

Material and methods

In the present experiments, the small amount of cysteine in leukocytes was disregarded, whereas the larger amounts of cysteine (S-H)

and, to be certain, also the cystine (S-S) in all available media had to be circumvented. After several attempts with various media, it was found that the mercury treatments could be performed with reproducible results if the cells were exposed a limited time to the mercurials dissolved in a balanced salt solution. Before and after the treatment the cells were kept in ordinary culture medium. The two mercury compounds tested were methyl mercury chloride (MMC) and methoxyethyl mercury chloride (MOEMC).

The following procedure was employed: Leukocytes from peripheral blood were cultured according to a slight modification of the technique of MOORHEAD *et al.* (1960). The blood was taken from different healthy donors. The cultures from some of the donors had always very few mitoses and could not be used. It was ascertained that the differences in response to the mercurials was not greater among the different donors than among samples from one of them. The leukocytes were separated by sedimentation for one hour in tubes held in ice water, followed by centrifugation at 600 rpm for 5 minutes and at 1000 rpm for further 5 minutes. Two ml of leukocyte-laden plasma were added to 6 ml of Earle-Eagle's medium with antibiotics and 0.3 ml of Bacto's phytohemagglutinin. This medium was selected partly because its composition of inorganic salts is the same as the BSS used, and partly because it contains no cysteine (and thus no S-H groups), only the S-S groups of cystine. The leukocyte suspension was distributed into roller tubes, 1.5 ml into each, incubated for 71 hours at 37°C in tightly capped tubes. If necessary for the pH value, the caps were loosened and the tubes placed in CO₂ atmosphere for a few hours. Before treatment, the cultures were transferred into centrifuge tubes, the cells spun down at 1000 rpm for 10 minutes, the supernatant poured off, and the cells resuspended in 1.5 ml of Earle's balanced salt solution containing the series of concentrations of the mercurial to be tested. The time of treatment was 1 hour at 37°C. After this time hypotonic treatment was performed for 10 minutes by adding the triple amount of distilled water to the medium. The cells were spun down, the supernatant discarded and the bottom fixed in 9 parts of 60% acetic acid and 1 part of 1 N

hydrochloric acid for 15–20 minutes. The cells were then squashed manually in 2% orcein in 60% acetic acid. No colchicine treatment was performed except in specific controls. Other controls were taken of the cells in the salt solution and in the medium. Several experimental series were made.

Observations

The results from 3 series with MMC and 1 series from MOEMC are summarized in Table I, from which it is seen that the two compounds tested gave comparable effects. In high concentrations, 200 to 2000 $\times 10^{-6}$ M, almost all mitoses exhibited strong toxic or lethal effects. The chromosomes became clustered in the centre of the cell as a dense lump, or were arranged into a ring around an open area (Fig. 1 a, b). In these cases the centromeres were directed towards the centre of the cell and the chromosome arms arranged radially. This may mean a c-mitotic action, thus a vital reaction, before the toxic action killed the cell. This recalls certain pictures after colchicine treatment of ascites cells (LEVAN 1954, Fig. 5 d, e, p. 14) and indicates strong toxic action, in the present case usually even fixation to the cells. The entire cell content was hard and less yielding to squashing than normally. There was a tendency of the plasm to stain more deeply than usual, at the same time as the chromosomes were less stainable. In these fixing concentrations, the chromosomes showed considerable damage and also often signs of heterochromaty: darker and lighter regions alternating (Fig. 1 a, c, f).

In the next lower concentration zone, 10 to 100 $\times 10^{-6}$ M, c-mitosis predominated. The treatment was often extremely favorable for obtaining a great number of well-spread metaphases with unusually clear chromosomes. Contraction was variable, often quite strong (Fig. 1 e, g) but sometimes ideal for morphologic analysis (Fig. 1 h), and it is quite likely that certain mercury treatments could be worked out that would be good substitutes for colchicine treatment in improving the appearance of metaphase chromosomes. As the concentration of the mercury compounds was lowered, the fraction of complete c-mitosis decreased and at or below 1×10^{-6} M control level was reached. This level was fairly high, around

Table 1. The action of two organic mercury compounds, methyl mercury chloride (MMC) and methoxy ethyl mercury chloride (MOEMC) on human short-term leukocyte cultures

Time of treatment: 1 hour, 100 cells counted for each value. For MMC the values are means of three experimental series.

	Concentration Mol $\times 10^{-6}$	MMC			MOEMC		
		Strong toxicity	C-mitosis	Normal mitosis	Strong toxicity	C-mitosis	Normal mitosis
Treatments	2000	100	0	0	—	—	—
	200	95	5	0	89	11	0
	100	31	60	9	54	29	17
	50	16	69	15	15	50	35
	20	12	66	22	2	42	56
	10	5	60	35	0	37	67
	5	0	42	58	0	32	68
	2	0	37	63	0	21	79
	1	0	22	78	0	15	85
	0.5	0	10	84	0	15	85
	0.2	0	10	90	0	17	83
	0.02	0	12	88	0	18	82
Controls	B.S.S.	0	9—13	87—91	0	17	83
	Medium	0	10—13	87—90	—	—	—
	Colchicine 0.2 μ g/ml	0	88—92	8—12	0	88	12

10 %, probably depending on the unnatural environment of the cells. It was also noticed that deviations in the pH value of the medium caused a more sluggish mitotic activity and an increase in the incidence of c-mitosis.

Signs of chromosome breakage were observed occasionally (Fig. 1 d) but always in a very low frequency. No attempts were made to estimate this frequency, but it was certainly far below 1 % of the mitoses.

The present experiments show that short-term human leukocyte cultures may be utilized as a convenient system for studying the toxic and c-mitotic activities of organic mercury compounds. In comparison with the *Allium*-test system (FISKEJÖ 1969), the compounds usually gave somewhat lower threshold values.

Summary

The action of two organic mercury compounds on short-term human leukocyte cultures was studied in a series of concentrations, from killing concentrations down to concentrations

with no visible effect. By transferring the cells into a balanced salt solution for the period of treatment, the interference of cysteine S-H and cystine S-S groups, always present in culture media, was avoided, allowing the c-mitotic effects of the mercury compounds to be studied.

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Fig. 1. Mitoses of human leukocytes under the influence of organic mercury halogenides; b: 200, c, h: 20, d, g: 10×10^{-6} methyl mercury chloride; a, c, f: 200×10^{-6} methoxyethyl mercury chloride. The higher concentrations (a, b, c, f) give strong toxic, probably fixing effects with sometimes chromomere-like structures (a, c, f). Weaker concentrations (d, e, g, h) induce scattered c-mitosis, sometimes with strong chromosome contraction; very rarely evidence of chromosome breakage were seen in anaphases.—Acetic orcein squashes, $\times 1400$.

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Brief reports

GEIRID FISKESJÖ:

The effect of two mercury compounds on lysogenic *E. coli* K39 (λ)

(Received July 9, 1971)

With a lysogenic strain of bacteria, and with a method to measure induction of phages, one can at the same time observe the effects of a certain chemical on both bacteria and phages.

There are not many chemicals tested in this way. ZETTERBERG (1969) found that out of the two chemosterilants, TEPA and HEMPA, only TEPA had the ability to induce lysis in the lysogenic *E. coli* K12 (5).

Materials and methods

The chemicals tested are the same mercurials as tested on *Allium* cells (FISKESJÖ 1969) and on human leukocytes (FISKESJÖ 1970), Methyl mercury chloride (MMC) and methoxyethyl mercury chloride (MOEMC). The test organism *E. coli* K39 (λ) and the indicator strain K49 were received from the laboratory of prof. G. Bertani, Stockholm.

The method is a modification of the standard method used to induce lysis in lysogenic bacteria with UV-light (WEIGLE and DELBRÜCK 1951). Instead of the UV-light the material is exposed to the chemicals as follows:

The culture medium is discarded after centrifugation and the material is resuspended in a new medium in which a certain mercurial is dissolved. In this case the time of treatment was one hour, of which the last 15 minutes were used for centrifugation — the supernatant again being discarded and the material resuspended and diluted in 10^{-2} M MgSO_4 (Mg-ions are necessary for the adsorption of lambda).

Some of the spontaneously induced phages ("free phages") are lost by discarding the supernatants, but this procedure is the same for the controls and may be left out of account. The phages that might be induced from the mercurials are still not liberated and could not be lost in this way.

With ordinary plating of the bacteria and phages the effect of the mercurials could be cal-

culated. The strain *E. coli* K49 S^r was used as indicator for the phages — the lysogenic strain K39 being streptomycin-sensitive.

The amount of "free phages" was tested by adding streptomycin at the same time as the indicator. When measuring the proportion of induced phages the streptomycin was added by spraying two hours after incubation.

The culture medium was a full medium based on tryptons and sodium chloride. It can be assumed that there are no free amino acids in this medium and consequently no free sulphur amino acids with sulphhydryl groups which could interfere with the action of the mercurials (RAMEL 1969), as was the case with the medium for human leukocytes (FISKESJÖ 1970). Therefore, the treatment of the bacteria and phages was performed in the usual culture medium, in which the different concentrations of mercurials were dissolved.

Observations and discussions

Table 1 and 2 give the results for MMC and MOEMC respectively. The controls are variable because of the different concentrations of bacteria at the beginning of the treatment, and every treatment has its own control. The columns for "all phages" give the values for both spontaneously induced phages ("free phages") and phages induced by the mercurials. The figures for "all phages" in the treated material showed no more increase of phages than did the figures for "all phages" in the control. These small increases are due to the spontaneously induced phages during the two hours of incubation without streptomycin. Accordingly, the mercurials in the concentrations used had the ability to induce the phage lambda in the lysogenic *E. coli* K39.

The effect measured by this method for the different treatments only showed the killing or the inactivation of bacteria and of phages in different amounts.

The concentration of the mercurials were

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Table 1. Effects of MMC on bacteria and phages in *E. coli* K39

Conc. $\times 10^{-6}$ M	Bacteria/ml		Phages/ml, control		Phages/ml, treated	
	Control	Treated	"All phages"	"Free phages"	"All phages"	"Free phages"
2000	7.5×10^7	0	2.1×10^5	1.7×10^5	1.7×10^3	1.6×10^3
200	6.8×10^7	5.8×10^6	5.2×10^5	4.8×10^5	1.0×10^4	1.0×10^4
100	9.7×10^7	1.0×10^7	1.1×10^6	9.0×10^5	4.0×10^4	3.8×10^4
50	2.4×10^7	1.7×10^6	2.7×10^5	2.4×10^5	1.0×10^4	1.1×10^4
20	9.5×10^7	9.9×10^6	5.8×10^5	5.5×10^5	1.2×10^4	9.0×10^3
10	5.2×10^7	1.3×10^6	4.6×10^5	4.4×10^5	7.5×10^3	5.8×10^3
5	9.8×10^7	5.2×10^7	6.0×10^5	5.0×10^5	4.8×10^4	1.8×10^4
2	7.7×10^7	6.0×10^7	4.9×10^5	4.1×10^5	9.2×10^4	7.5×10^4
1	8.6×10^7	8.6×10^7	5.3×10^5	4.6×10^5	5.1×10^5	4.2×10^5
0.5	2.6×10^7	2.5×10^7	2.1×10^5	1.7×10^5	2.0×10^5	1.6×10^5

Table 2. Effects of MOEMC on bacteria and phages in *E. coli* K39

Conc. $\times 10^{-6}$ M	Bacteria/ml		Phages/ml, control		Phages/ml, treated	
	Control	Treated	"All phages"	"Free phages"	"All phages"	"Free phages"
200	1.2×10^8	4.2×10^5	5.8×10^5	5.2×10^5	3.2×10^3	3.0×10^3
100	5.0×10^7	2.8×10^6	5.2×10^5	3.7×10^5	2.0×10^3	1.8×10^3
50	1.3×10^8	8.5×10^6	6.0×10^5	4.5×10^5	8.8×10^3	8.4×10^3
20	2.5×10^7	8.5×10^5	1.5×10^5	1.0×10^5	2.3×10^3	2.2×10^3
10	4.2×10^7	1.0×10^6	2.6×10^5	1.5×10^5	1.7×10^3	9.0×10^2
5	3.5×10^7	2.2×10^7	3.2×10^5	2.8×10^5	4.2×10^4	9.3×10^3
2	5.3×10^7	5.2×10^7	3.0×10^5	2.5×10^5	2.5×10^5	1.4×10^5
1	2.7×10^7	2.9×10^7	2.6×10^5	2.2×10^5	2.3×10^5	2.0×10^5
0.5	1.6×10^7	1.7×10^7	2.3×10^5	8.9×10^4	2.3×10^5	9.0×10^4

mainly from 0.5 to 200×10^{-6} M. The concentration of 2000×10^{-6} M for MMC was also tested. All the bacteria were killed by this treatment but not all the phages. The values obtained after MMC-treatment infer that the threshold concentrations are between 1 and 2×10^{-6} M. The threshold level for the effects of MOEMC seems to be the same as for MMC for the phages, but higher for the bacteria, being between 2 and 5×10^{-6} M.

Fig. 1 shows graphically in what way the bacteria and the phages survive the treatments.

The threshold values for the effects of MMC and of MOEMC for bacteria and phages are almost the same as for *Allium* cells and for human leukocytes as shown in Table 3. The leukocytes are slightly more sensitive and the bacteria slightly

more resistant to the mercurials than the other tested organisms. However, the phages are more resistant in the highest concentrations of the treatments used.

Summary

None of the mercurials MMC and MOEMC had the ability to induce the phage lambda in the lysogenic *E. coli* K39 (λ). The survival of the bacteria and phages after different treatments with MMC and MOEMC was measured. The effects of these mercurials on the lysogenic *E. coli* K39 were compared to the effects of the same mercurials on two other types of cells: root tip cells of *Allium cepa* and human leukocytes. In all the concentrations MMC and MOEMC gave

Table 3. Effects of mercurials on different cell systems

	Lowest concentration with growth disturbances $\times 10^{-6}$ M				Highest concentration with growth disturbances $\times 10^{-6}$ M			
	Bacteria	Phages	Allium	Leukocytes	Bacteria	Phages	Allium	Leukocytes
MMC	2	2	2	1	1	1	1	0,5
MOEMC	5	2	2	2	2	1	1	1

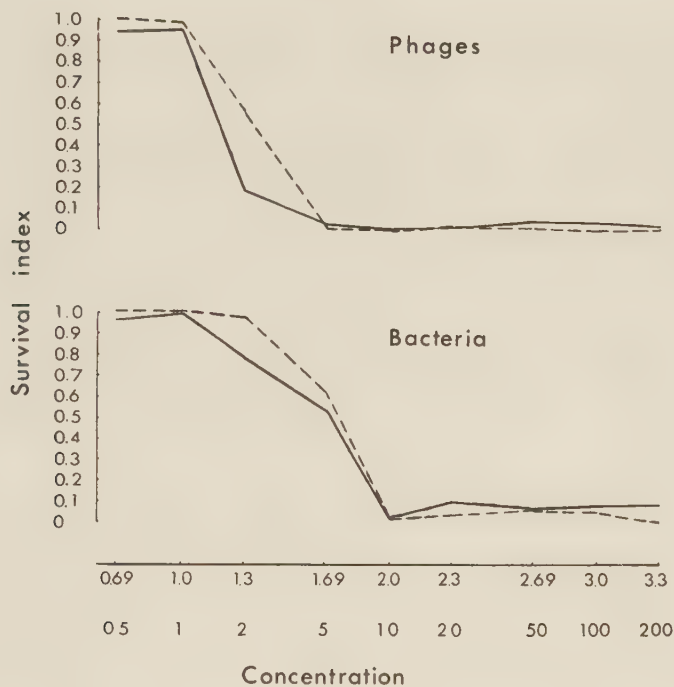


Fig.1

Fig. 1. The relationship between mercury-treated material and controls for different concentrations of MMC (whole line) and of MOEMC (broken line). The abscissa gives the logarithmic values of the concentrations of the mercurials ($\times 10^{-7}$). The corresponding values as given in the tables are pointed out below ($\times 10^{-6}$).

similar results in the three different cell systems.

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Two organic mercury compounds tested for mutagenicity in mammalian cells by use of the cell line V 79-4

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FISKESJÖ, G. 1979. Two organic mercury compounds tested for mutagenicity in mammalian cells by use of the cell line V 79-4. – *Hereditas* 90: 103–109 Lund, Sweden. ISSN 0018-0661. Received October 2, 1978

Organic mercury has been an environmental problem for many years. In spite of the high toxicity of organic mercury compounds and their accumulation ability in living organisms, the spreading in the environment has been steadily increased by human activities.

In this study, the two organic mercuries, methyl mercury chloride (MMC) and methoxyethyl mercury chloride (MO), have been tested for mutagenicity in mammalian cells by use of the Chinese hamster cell line V 79-4 with two genetic markers: the locus *aza* for nucleic acid synthesis and the locus *oua* concerning the cell membrane. High toxic effects were obtained by MMC and by MO, beside a certain low mutagenicity in both of the tested loci; MO being slightly more mutagenic than MMC.

The very narrow dose response curves were found to lie close to the threshold values for toxicity.

Since the two tested mercury compounds had lower threshold values in the mutagenicity tests with V 79 cells than in toxicity tests with four different materials (*Allium*, human lymphocytes, *E. coli* K39 (λ) and V 79 cells), the mutagenicity test may generally be the more sensitive tool to evaluate biological effects of chemical compounds.

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Mercury exists in many forms in natural sources throughout the world. Most of the mercury compounds are now deposited as sediments in lakes, rivers and oceans; the mercury burden of lakes and rivers for instance has increased 3 to 4 times pre-man levels (Wollast et al. 1975). Dentistry provides the main part of the "intentional" water-borne mercury pollution, chloralkali industry is the main supplier of "intentional" air-borne mercury pollution, whereas "unintentional" releases of mercury originate from mining activities, agriculture, burning of garbage and batteries, lime and cement, power plants and crematoriums (National Swedish Environment Protection Board 1978).

By means of bacteria, part of the inorganic sedimenting mercury is transformed into organic forms of mercury compounds (JENSEN and JERNELÖV 1974). Some of these organic forms are soluble in water and accumulate, in the form of monomethyl mercury, in water organisms (BOETIUS 1960; HANNERZ 1968). This is one of the two mercury forms used in the present study. Also the other mercury form investigated here,

methoxyethyl mercury, is transformed at low pH into methyl mercury (alkyl mercury) (JENSEN and OLSSON 1971).

From about 1940 to 1965, alkyl mercury was used as a fungicide for seed treatment in Sweden. When, in the early sixties, it was found that alkyl mercury caused the death of many birds, both seed-eaters and birds of prey (BORG 1958; BORG et al. 1965), the Poison Board (Giftnämnden) in 1965 decided to prohibit the use of alkyl mercury (because of its high accumulation power) and, as a substitute, to allow restricted use of the chemically less stable alkoxyalkyl mercury compounds.

In 1967, however, ESBO et al. recommended even greater restrictions, their pleading being for non-mercury treatments of seed. Similar conclusions are expressed by D'ITRI (Panel on Mercury 1978): "mercury compounds have no metabolic function", and "all such contamination must be regarded as undesirable and potentially hazardous".

Toxicity of the different organic mercury compounds has been reported on at many times (for review, see D'ITRI 1972; Panel on Mercury 1978;

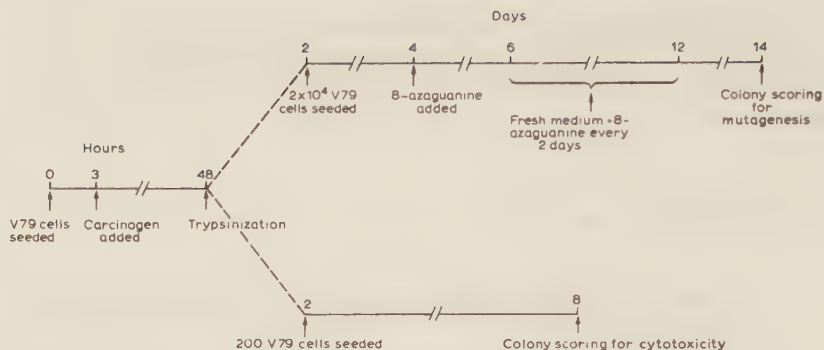


Fig. 1. Schematic outline of the experiment (from HUBERMAN 1975; with permission from author and Elsevier/North-Holland Biomedical Press, Amsterdam).

and RAMEL 1972). Teratogenic effects also have occurred (e.g. in the Minimata disease) but the few trials made to establish cancer as an effect of methyl mercury have failed (for review, see Panel on Mercury 1978).

Mutagenicity, however, has been shown in *Drosophila*. A slight increase in the frequency of X-linked recessive lethals was reported by RAMEL (1972), and, recently, more significant increases in X-linked recessive lethals after exposure of *Drosophila* to mercury-*p*-toluene-sulphanamide have been observed (MATHEW and AL-DOORI 1976).

Since a test for mutagenicity of organic mercury compounds in mammalian cells had not earlier been performed, it became the purpose of the present investigation.

Materials and methods

Two organic mercury compounds, methyl mercury chloride (MMC) (Casco) and methoxyethyl mercury chloride (MO) (Plantex), were tested in a system with the Chinese hamster cell line V 79-4. Cells were obtained from E. Huberman, Israel, and the test method followed the main outlines presented by HUBERMAN and SACHS (1974) (Fig. 1), and included a cell-mediated form and a direct form. In the cell-mediated test, chemicals are metabolically activated by another cell layer before the measuring of mutagenicity on V 79-4. In the direct test, chemicals are directly applied to the V 79-4 cells without a mediating metabolizing cell layer.

The tested mercuries showed effects in direct tests on the V 79-4 cells; preliminary experiments showed that the cell-mediated test did not alter the conclusions obtained from V 79-4 alone. Only the direct tests will be reported here.

Two genetic markers *aza* and *oua* are used to select mutants; the *aza* locus is connected with the nucleic acid synthesis, the *oua* locus concerns the cell membrane. Cell variants resistant to ouabain have been isolated from human fibroblasts by MANKOVITZ et al. (1973) and from mouse and hamster cells by BAKER et al. (1974). The *aza* locus has been found to be shared by many mammalian cells; *aza* has been reported to be an X-linked gene in man (NYHAN 1968) as well as in horse (OHNO 1973), mouse (EPSTEIN 1972), the field vole (COOK 1975) and the Chinese hamster (CHU et al. 1969).

Cells were grown in Dulbecco's medium H 21 (Gibco) with 10% fetal calf serum and standard amounts of penicillin and streptomycin. Per 50 mm plastic petri dish (Falcon) 5 ml medium were used, and the cultures were incubated at 37°C in a humidified incubator supplied with a constant flow of 10% CO₂ in air.

The experiments were performed as follows: Cells in good condition were treated with 0.25% trypsin solution for 30 min. For toxicity tests the obtained single-cell suspension was spread with 200 cells per petri dish. For mutagenicity tests with the *aza* locus, 2×10^4 cells and, in the *oua* tests, 10^5 cells were spread per petri dish. For each concentration in a treatment 16 petri dishes were used.

After one day, treatment was performed for 3 h

with test chemicals in different concentrations added to the medium. Then the growth medium was freed from test chemicals, and the cells were allowed 2 days for the possible mutants to manifest themselves. For the screening for *aza*^R mutants, 8-azaguanin (Sigma) (30 µg/ml medium), and for *oua*^R mutants, ouabain (Sigma) (1 mM/ml medium) were then added. The medium with 8-azaguanin was replaced every 2 days. The ouabain medium was originally supposed to require no change (HUBERMAN and SACHS 1976) but, under the present laboratory conditions, best results were obtained with one change of the ouabain-containing medium about one week after the first ouabain addition.

Cloning efficiency was determined from counts of the Giemsa-stained colonies in the toxicity test, 6–8 days after the start of the experiment. The number of mutants after different mercury treatments was determined, for the *aza*^R mutants, after 12–14 days and, for the *oua*^R mutants, after 16–17 days.

As a positive control for mutagenicity N-methyl-N-nitro-N-nitrosoguanidin (Sigma) was used in the concentration 0.5 ppm, and as a negative control the medium itself was used. Methyl mercury chloride was tested in the concentrations 0.1–0.5 ppm, and methoxyethyl mercury chloride from 0.05 to 0.3 ppm.

Results

Both MMC and MO seemed to have a certain but weak mutagenicity, and both were highly toxic. Repeated experiments always gave closely similar results; MO being somewhat more toxic and also slightly more mutagenic than MMC.

Table 1 shows the results for three concentrations of each of the two mercuries, for one concentration of the positive control nitrosoguanidin (Ng) and, finally, for the negative control. The table shows that the *oua* locus is less mutable than the *aza* locus, the ouabain-resistant mutants after mercury treatment mostly being too few to give a mean exceeding the zero value even when calculated on 10⁵ survivors. The graphical presentation of the data (Fig. 2) was, therefore, restricted to the azaguanin results.

Fig. 2 shows that the curves for dose response to the mercuries are limited to very narrow concentration ranges; below 0.1 ppm for MMC and 0.05 ppm for MO no visible effects could be

Table 1. Direct test on V 79-4 cells after treatment with the two organic mercuries MMC and MO (three concentrations), medium control (C), and positive control (Ng, one concentration). All values are based on a mean from 16 dishes

Treatment (μg/ml)	Survival %	Mutant frequency per survivors	
		<i>aza</i> ^R per 10 ⁴	<i>oua</i> ^R per 10 ⁵
MMC			
0.5	30.5	9.3	0
0.3	68	1.9	0
0.1	80.5	1.4	0
MO			
0.3	30	7.3	3.7
0.1	62.5	2.4	1.0
0.05	76	2.4	0
C	78.5	0.9	0
Ng 0.5	10.5	140	298

noticed, neither for toxicity, nor for mutagenicity; and above 0.5 ppm for MMC and 0.3 ppm for MO there is practically no survival.

In order to increase the precision, especially for the *oua* locus, a second experiment was designed to obtain higher numbers of mutants. Whereas the number of seeded cells for the Ng treatment was still kept at 2×10^4 for the azaguanin test and at 10⁵ for the ouabain test, the numbers of seeded cells for the mercury treatments were increased to 10⁵ resp. 10⁶. Only two concentrations within the ranges for mutagenic effects of each of the mercuries were investigated. In the positive control, Ng, because of its instability in medium, was added at 1 min or 15 min after being diluted in medium.

From the two positive controls with Ng treatments somewhat different results were obtained, but both treatments showed extremely high mutation rates (Fig. 3 and Table 2). For the mercury treatments the higher amount of seeded cells made possible more reliable estimates of the mutation rates, even for the ouabain locus. When MMC and MO in the same concentrations are compared (0.2 ppm) the MO treatment seems to be more toxic and more mutagenic than MMC.

Some mutant colonies were cultivated for about 1 month without 8-azaguanin or ouabain, whereafter the selecting drug was again added. During further cultivation the mutant cells showed a continued resistance to 8-azaguanin or ouabain.

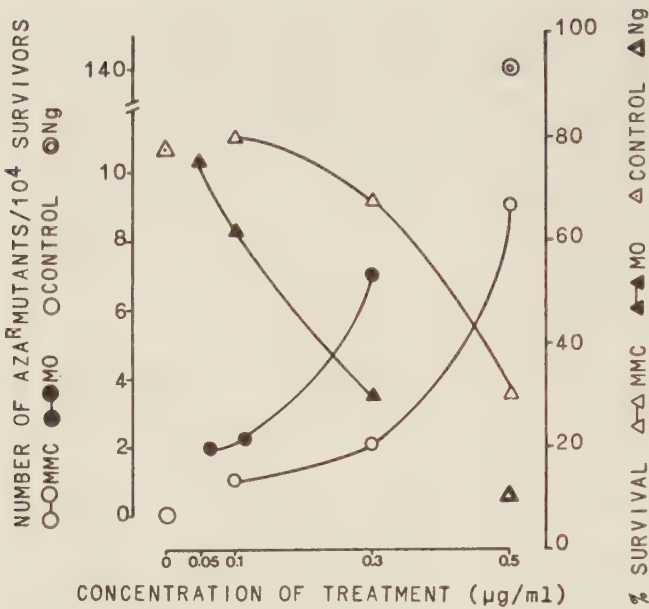


Fig. 2. Dose response in mutability from sensitivity to resistance to 8-azaguanin when V 79-4 cells were treated with the organic mercuries MMC and MO.

Discussion

The threshold values for the effects of the mercuries MMC and MO in this test system lie close to the threshold values for effects in three previously used systems (FISKESJÖ 1969, 1970,

1971). A comparison between the four different test systems shows that the V 79 values for MMC are very close to the previously obtained threshold values; the V 79 values for MO seem to be lower than in other systems (Table 3).

Bacteria, phages and *Allium* compared with V

Table 2. Direct test on V 79-4 cells after treatment with the two organic mercuries MMC and MO (two concentrations), medium control (C), and positive control (Ng, 1 min and 15 min after the Ng was diluted in medium). All values are based on a mean from 16 dishes

Treatment (μg/ml)	Survival %	Numbers of <i>aza</i> ^R mutants		Numbers of <i>oua</i> ^R mutants	
		per seeded cells	per 10 ⁴ survivors	per seeded cells	per 10 ⁴ survivors
MMC					
0.4	18	164/10 ⁵	91.1	23/10 ⁶	12.8
0.2	68	125/10 ⁵	18.3	10/10 ⁶	1.5
MO					
0.2	44	135/10 ⁵	30.7	22/10 ⁶	5.0
0.1	68	121/10 ⁵	17.8	31/10 ⁶	4.6
C	95	41/10 ⁵	4.3	0/10 ⁶	0
Ng 0.5					
1 min	15	134/2 × 10 ⁴	446.6	45/10 ⁵	300.0
15 min	29	187/2 × 10 ⁴	275.0	53/10 ⁵	182.8

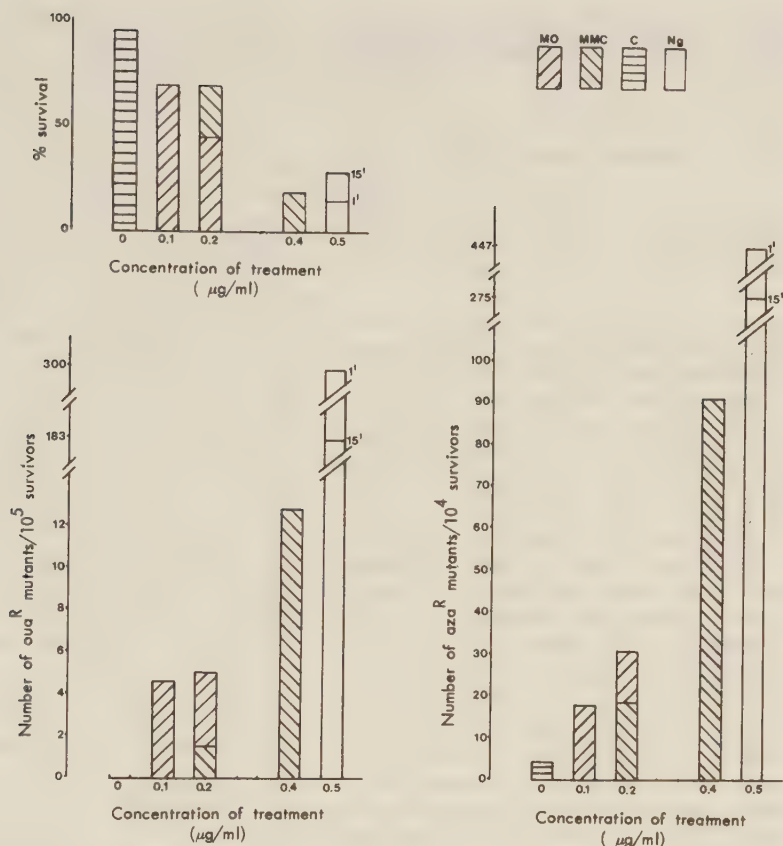


Fig. 3. Mutagenic effects of the organic mercuries MMC and MO, measured by the ability to mutate the loci *aza* and *oua* in the cell line V 79-4 of Chinese hamster. MO is slightly more toxic and even more mutagenic than MMC (best seen after 0.2 μg/ml treatment, which is used for both MO and MMC).

79 cells may be slightly less sensitive to the mercury compounds. The deviating threshold values for the lymphocytes compared with the V 79 values may find their explanation in the control values for the lymphocytes (FISKESSJÖ 1970); in these controls always a certain amount of C-mitoses were found, probably because of cultivation difficulties for lymphocytes *in vitro*. Thus, the threshold values for the lymphocytes may in reality be lower and in better agreement with the V 79 values than indicated by the data in Table 3. The three former tests and also the cloning efficiency test for the V 79 cells may be considered toxicity tests. Compared with these toxicity tests,

even with the V 79 cells, the V 79 cells in the mutagenicity test show lower threshold values. This may mean that the mutagenicity test is more sensitive than the toxicity test.

In the experiment with the higher amount of seeded cells (Table 2), the rate of 8-azaguanin mutants was about ten times higher than in the preceding experiment with the smaller number of seeded cells (Table 1). The reason might be that the mutants survive better when they are not too few. More probably, however, at the high amounts of 8-azaguanin mutants per petri dish, a spreading of mutant cells by the change of the 8-azaguanin medium every 2 days, is a source of

Table 3. Threshold values for effects of MMC and MO in four different test systems. The lowest concentrations leading to growth disturbances are given in ppm

Test chemicals	Toxicity tests					Mutagenicity test V 79
	Bacteria	Phages	Allium	Lymphocytes	V 79	
MMC	0.5	0.5	0.5	0.25	0.2	0.1
MO	1.25	0.5	0.5	0.5	0.1	0.05

error. For the 8-azaguanin test a more convenient start number of cells should probably be only slightly higher than that used for Ng.

However, both MMC and MO possess high toxicity and also a low but clear mutagenicity. A clear correlation between mutagenicity and cancer has been indicated (EHRENBERG 1974; HUBERMAN 1975; DE SERRES 1976). Also mercury is known to be stored in living organisms and may cause mutations (and cancer?) even below the level for visible toxicity. When, in 1965, the methoxy mercury was preferred as a fungicide and the use of methyl mercury was stopped in Sweden, the argument was that, compared to MMC, MO was chemically less stable and also had less effect on the nervous system (BERGLUND et al. 1971). Since the present data indicate that both these mercury compounds are mutagenic even in mammalian cells, and since, moreover, the methoxy compound (MO) is slightly more mutagenic than the methyl compound (MMC), one may consider MO to be at least as dangerous as MMC. The conclusion should be that none of these compounds, as well as other mutagenic compounds, should be allowed to be spread in the environment.

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Mercury and selenium in a modified *Allium* test

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A modified *Allium* test is presented implying that from the start of the experiment series of onions are grown in the different liquids to be tested – without first letting the root tips sprout in pure water. For screening purposes, macroscopical observations after two days of treatment, documented with photographic evidence of the degree of growth retardation or stimulation, will often suffice.

For specific purposes, these observations should be supplemented by microscopic analysis, as has been done in the present work.

The two procedures of the *Allium* test were compared: The new version, with longer time of treatment, seemed to be a little more sensitive than the original method.

The specific aim of this study was to investigate whether sodium selenite has any effect antagonistic to methylmercury chloride poisoning, as has been reported in certain animal materials. No such effect was observed in the present experiments.

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The *Allium* test was introduced by LEVAN in 1938 and has been in frequent usage since then (for instance LEVAN 1939, 1940; LEVAN and TIJO 1948; LEVAN and LOTFY 1949). Furthermore, the symposium on "Evaluation of genetic risks of environmental chemicals" in Sweden 1972 recommended the *Allium* test as a routine screening test with many advantages, such as low cost, short test time, ease in handling, excellent chromosome conditions (Royal Swedish Academy of Sciences 1973). The procedure of the original test implied root tips grown out to a length of 1–2 cm in fresh water of known quality, and thereafter exposed to specific treatments followed by macroscopic and microscopic observations after a certain time.

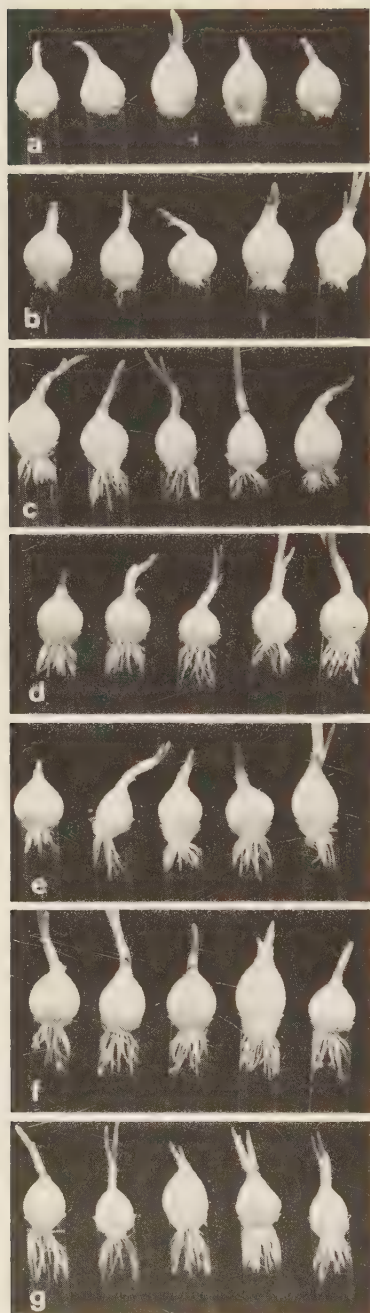
However, weak contaminations in naturally occurring water, as in water from rivers or other supplies of water for human use, often gave very little effect in the original form of the test. In order to detect even weak and unknown contaminations, a new procedure of the *Allium* test was designed (FISKESJÖ 1975, 1976), series of onions being placed directly in the water to be tested and the final observations being made after a few days. Since no initial treatment with pure water was included, this method of "treatment" is actually more similar to conditions in nature.

Even with amounts of toxic contaminations that by chemical analysis were defined as "small", this direct form of the test produced effects observable as differences in root length among the different experimental series of bulbs. With more severe toxic influences also the shape and colour of the root tips were affected.

For further extending the significance of the results, microscopic studies can be performed, but even photographs of the macroscopic materials have proven sufficient to reveal toxic effects when tracing the source of contamination of river water etc. The simplified form of the test has recently been useful in the testing of ordinary drinking water; it was demonstrated that different amounts of copper (originating from copper pipes) were correlated to different degrees of growth restriction of the *Allium* roots (FISKESJÖ 1978).

This new modification of the *Allium* test has also been convenient for studying the action of different concentrations of known chemicals. In the present work, the new modification of the test has been used in its widest form, also including microscopic investigation, as recommended in the original *Allium* test.

The effects of some organic mercury compounds have been studied earlier by means of



the original *Allium* test (FISKESJÖ 1969). Data from animal studies have suggested that selenium has a protective effect against methylmercury poisoning when given as sodium selenite in the diet to rats (PARIZEK and OSTADALOVA 1967; GANTHER et al. 1972) and also when present naturally in marine mammals (KOEMAN et al. 1973) or in the tuna (OHİ et al. 1976). However, the metabolic function of selenium and also the biochemical mechanism for selenium-methylmercury antagonism is not presently understood (O'DELL and CAMPBELL 1972; OEHME 1972; PANEL ON MERCURY 1978).

In the present investigation the *Allium* test was applied to see whether selenium has any protective effect against mercury poisoning in plants, and also to compare the two procedures of the *Allium* test.

Materials and methods

The material in the present experiments were equal-sized bulbs of the onion (*Allium cepa*, $2n=16$). Bulbs were chosen which had not yet started any growth of roots or green leaves and which were not infected by mould. Still it happened, even in the control material, that some onions grew poorly. Therefore, in order to be sure to have 5 uniformly growing bulbs, 6 or 7 were set up in each concentration of the test chemical and in the control. After two days, the 5 best onions in each series were used for the experiment.

At the start of the experiment, the yellowish outer coatings of the bulbs and the brownish bottom plate inside the ring of root primordice were carefully removed and the bulbs rinsed in control water. Thereafter they were placed into the test liquids on top of test tubes selected to fit the size of the bulbs. The same size of vessels were used in one entire experiment.

As a routine, water and test liquids were changed every day during the first three days; after that, only the evaporated amounts of liquid were replaced. All test liquids were freshly dissolved and diluted, and both the control water and the test liquids were stored at $+4^{\circ}\text{C}$ during

Fig. 1a-g. Growth retardation effects of sodium selenite after 2 days treatment in different concentrations; a: 1000 ppm; b: 100 ppm; c: 10 ppm; d: 5 ppm; e: 1 ppm; f: 0.5 ppm; g: control water. Five bulbs in each series of treatment.

the time of experiment. Before each change the test liquids were brought into room temperature about an hour in advance.

After two days of cultivation, and in some cases also after five days, the series of bulbs were photographed and cytological preparations for microscopic studies were prepared according to the ordinary squash procedure: Fixation and maceration/hydrolysis in a mixture of 9 parts 45% HAc and 1 part 1N HCl at 50°C for 5 min, followed by squashing in 2% orcein in 45% HAc. From each of the five bulbs of a series, one root tip was taken and used for preparation. From each slide 100 mitoses were analysed and the mitotic index was scored from 200 cells.

Fresh tap water from the Institute of Genetics, in which copper pipes are not used for the cold water, was used as standard "medium" for controls and for preparing and diluting the test chemicals.

The mercury concentrations were the same as in an earlier study of organic mercury compounds by the original Allium test (FISKESJÖ 1969). These concentrations ranged from 0.01 ppm to 50 ppm of mercury in the form of methylmercury chloride (MMC, Casco). The monomethyl mercury was chosen because it is the compound into which bacteria transform most of the waste mercury in the environment (JENSEN and JERNELÖV 1969) and being easily taken up by plants (DOLAR et al. 1971).

Preliminary tests with sodium selenite ($\text{NaSeO}_3 \cdot 5\text{H}_2\text{O}$, Sigma) comprised a concentration range of 0.5–1000 ppm of selenite. For the combined treatments with selenium (Se) and mercury (Hg), a sodium selenite concentration was chosen just below the concentration 10 ppm which by itself gave growth retardation.

Three series were performed for the co-treatment experiment: (1) Hg and Se in equal amounts from 0.5 to 125.5 ppm for each; (2) different concentrations of Hg from 0.01 ppm to 10 ppm Hg and co-treatment with 1 ppm Se for each of the different Hg concentrations; (3) fivefold excess of Se, and Hg in concentrations from 0.01 to 1 ppm.

Results and discussion

1. Effects of Se

To determine the effect of Se on growing Allium roots an experiment with the new modification

of the Allium test was set up with pure sodium selenite, as illustrated in Fig. 1. The series of photographs of this figure can be read as a table: In each series of 5 onion bulbs there is an easily observable macroscopic mean response to the treatment, the root tips showing an increasing degree of growth retardation at increasing concentrations of the chemical.

At 1000 ppm concentration, no roots grew out at all; at 100 ppm the roots grew, although poorly; at 10 ppm there was still some growth inhibition; at 5 ppm and 1 ppm there were only weak disturbances; and at 0.5 ppm, finally, no difference was seen in comparison with the control series.

Similar degrees of toxic effects were revealed by the microscopic studies (Table 1): At 10 ppm and higher the number of dividing cells had decreased and at 100 ppm the mitotic index was very low. No specific chromosome damage was induced by the selenium treatment: There were some stickiness and delayed anaphases and also a low frequency of vagrant chromosomes during mitosis, whereas c-mitosis occurred only to a very low extent.

The commonest types of chromosome disturbances in the Se experiment are shown in Fig 2, viz. anaphases with different types of bridges (a–c), vagrant chromosome (a), and delayed anaphase (d). The chromosome bridges were usually due to separation difficulties at specific points of the chromosomes rather than to general stickiness. Pseudochiasmata were seen occasionally, a typical one being pictured in Fig. 2c.

2. Effects of Hg and of Hg + Se

The next part of the experiment was to investigate whether Se, in co-treatments with Hg, could counteract the toxic effects of Hg.

The results were negative when treatments with equal amounts of Hg and Se were applied in the concentrations of 100, 40, 10, 4, 1, 0.4 and 0.1 ppm. Also the second co-treatment experiment, with 1 ppm of Se together with varying amounts of Hg, gave negative results. In Fig. 3, the macroscopic results of these co-treatments are compared with the results of the corresponding treatments with Hg alone. In no case did the addition of Se effect any improvement of the growth of the Allium roots.

Fig. 4 shows the third co-treatment experiment with certain concentrations of Hg and fivefold excess of Se. Not even here could any clear

Table 1. Cytological effects of different treatments with sodium selenite, ($\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$, mol wt = 263.04); methylmercury chloride, (MMC, mol wt = 251.10); and combinations of both

For microscopic studies, slides were prepared from each of the 5 bulbs in a series with one root tip on each slide. The mitotic index was based upon 1000 cell counts (200 cells from each slide) and for the classification of cytological effects, some 500 cells were scored (about 100 cells per slide)

Treatment	Microscopic effects in %														
	Selenium in molarity $\times 10^{-6}$	Se in ppm	Mitotic index in %	No. of counted cells	Microscopic effects in %							Frag- ments	Multipolar anaphases	Polyploid C-mitosis	
					Normal meta- phases	Normal ana- phases	"Delayed" ana- phases	C- mitosis	Sticky stages of mitosis	Bridges	Vagrant chromo- somes				
<i>Sodium selenite</i>															
3803	1000	300.3	0	0	47.9	42.3	2.1	1.4	3.5	2.1	0.7	0	0	0	
380	100	30	0.6	142	48.1	32.8	10.3	1.3	5.8	0.7	1.0	0	0	0	
38	10	3	4.3	399	53.4	30.8	12.0	0.6	1.8	1.0	0.4	0	0	0	
19	5	1.5	4.7	500	45.6	39.6	10.0	0.2	1.4	2.8	0.4	0	0	0	
3.8	1	0.3	4.6	500	51.4	37.2	7.0	0.6	2.2	1.2	0.2	0	0	0	
1.9	0.5	0.15	4.2	500	54.6	34.6	8.0	0.2	1.2	0.6	0.8	0	0	0	
0.38	0.1	0.03	4.3	500	66.8	20.2	7.6	1.0	2.6	1.8	0.0	0	0	0	
0.19	0.05	0.01	4.8	500	56.8	35.5	5.0	0	1.2	0.8	0.2	0	0	0	
Control			4.3	500											
<i>MMC</i>															
50	12.5	10	0	0	0	0	0	0	(100)	0	0	0	0	0	
20	5.0	4	0	2	0	0	0	(90)	(10)	0	0	0	0	0	
5	1.25	1	0.2	10	0	0	0.4	81.9	16.1	0	0	0	0	0	
2	0.5	0.4	1.6	254	1.6	0	0	50.1	15.6	0.3	3.1	0	2.3	0	
0.5	0.125	0.1	2.9	353	12.2	13.9	2.6	61.6	3.8	0.2	1.6	0	13.6	0	
0.2	0.05	0.04	5.2	500	11.8	5.8	1.6	0.6	3.1	0.9	0.7	0	0	0	
0.05	0.012	0.01	3.7	458	60.7	21.8	11.3	0.6	3.1	0.9	0.7	0	0	0	
Control			4.3	500	50.8	44.6	2.2	0	1.2	1.0	0	0.2	0	0	
<i>Hg + Se</i>															
1 + 5			0.7	100	0	0	1	57	41	0	1	0	0	0	
0.4 + 2			1.6	262	1.5	0	0	83.2	9.6	0	0	0	0	5.7	
0.1 + 0.5			2.5	258	5.8	0	0	78.3	14.3	0	0.4	0	0.8	0.4	
0.04 + 0.2			2.9	253	13.0	0	0.4	53.4	15.0	0	0	0	18.2	0	
0.01 + 0.05			3.1	342	43.0	30.1	12.9	0.3	11.1	1.7	0.6	0	0	0.3	
Control			4.8	488	53.5	38.1	5.6	0.2	2.0	0.2	0.4	0	0	0	

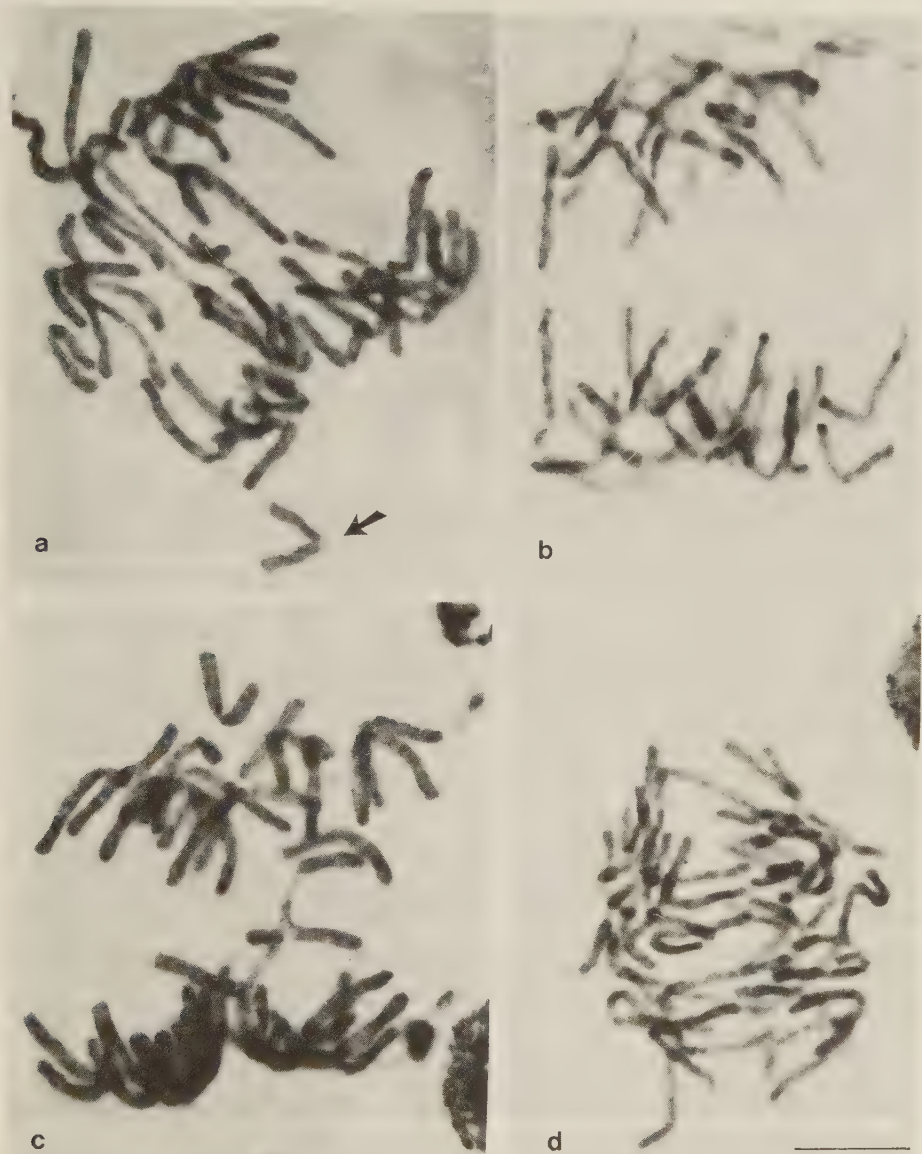
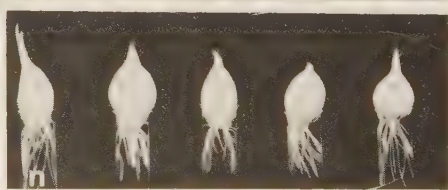
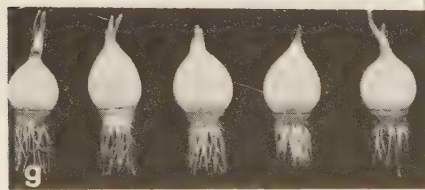
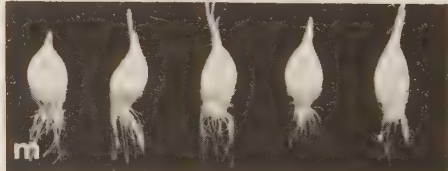


Fig. 2a-d. Microscopical effects of sodium selenite; a-c: sticky anaphases; d: delayed anaphase. Note the vagrant chromosome in a (arrow). a and b: 0.05 ppm Se; c: 10 ppm Se; d: 0.05 ppm Se. Scale = μ m.



improvements of growth be observed. Thus, in the growing *Allium* roots no effects of Se antagonistic to Hg poisoning were demonstrable. These negative results may mean that the metabolism and mode of storage of Se, and probably also of Hg, are different in plants and in animals.

The microscopic investigation (Table 1) showed that in contrast to the selenite the methylmercury chloride had c-mitotic effects as its main effects: c-metaphases, c-anaphases and multipolar anaphases (Fig. 5). The mitotic index was lowered and stickiness was increased at higher concentrations in the treatment. Fragments (from chromosome breaks) were found only rarely (Fig. 5d). Macroscopically the c-mitotic effect was accompanied by the development of c-tumours in the root tips; the c-tumours were best seen after 5 days of treatment (Fig. 5a).

Table 1 also shows that Se gave effects that were not antagonistic but rather additional to those of Hg alone. Both the stickiness and the number of c-mitoses were somewhat higher after the co-treatment than after the separate treatments with Hg or Se. Polyploid c-mitoses were only found in the co-treatments.

From the chemical point of view, TSEN and COLLIER (1959) have investigated whether selenite can be an inhibitor of sulfhydryl enzymes. Finding no support for this hypothesis, they suppose that selenium toxicity "may be explained by the selenium-catalyzed oxidation of such co-factors as glutathione, coenzyme A and dihydrolipoic acid, and the resulting disturbances in intermediary metabolism".

This may partly explain why the selenite in the *Allium* test does not interfere with the effects of mercury, since it is known that mercury acts by binding to the sulfhydryl compounds (WACHTMEISTER 1971).

Contrary to this, SUGIURA et al. (1976) have

shown that the selenohydril compound binds methylmercury 100 times more tightly than the sulfhydryl group, so it still remains unclear why selenium can be antagonistic against Hg in animals but not in plants (as indicated by the present results in the *Allium* test).

It appears from these co-treatments that selenium may act in a different way in plants than in animals, and it is possible that several compounds exist with similar differences in action. Thus, the *Allium* test should be used only as a screening test and direct conclusions should be limited to plants. Undoubtedly, a broad program including different tests will widen our knowledge concerning which types of chemicals have similar effects in different test systems, and with such knowledge extrapolation of results may eventually be feasible.

3. Comparison between the original and the modified *Allium* test

When MMC was studied in the original *Allium* test (FISKESJÖ 1969), loss of turgescence (softening of the root tips) was one of the macroscopic characteristics recorded. The lowest concentration resulting in complete loss of turgescence was 50×10^{-6} M. After recovery in water for 24 hours the root tips remained soft and, thus, the cells had been killed by the treatment. In the modified test, the roots were killed by 2 days of treatment in the nearest lower concentration, 20×10^{-6} M.

In Table 2, these data have been recorded together with similar data on 3 other types of responses; c-tumours, c-mitoses and microscopic disturbances in general. The comparison of these highly approximate data from the two procedures of the *Allium* test indicates that in all responses the concentrations of the original

Fig. 3a-n. Growth retardation effects of mercury (Hg, given as methylmercury chloride) and of mercury + selenium (Se, given as sodium selenite) after 2 days treatment. Five bulbs in each series of treatment. To the left (a-g) the co-treatment series (Hg+Se) and to the right (h-n) only mercury treatment in the corresponding concentrations of Hg.

a: 4 ppm Hg + 1 ppm Se; h: 4 ppm Hg;
b: 1 ppm Hg + 1 ppm Se; i: 1 ppm Hg;
c: 0.4 ppm Hg + 1 ppm Se; j: 0.4 ppm Hg;
d: 0.1 ppm Hg + 1 ppm Se; k: 0.1 ppm Hg;
e: 0.04 ppm Hg + 1 ppm Se; l: 0.04 ppm Hg;
f: 0.01 ppm Hg + 1 ppm Se; m: 0.01 ppm Hg;
g: control water; n: control water.

This experiment was performed on two occasions; the control to the left (g) belongs to the concentrations of Hg (a-d and h-k), and the control to the right (n) belongs to the concentrations of Hg (e, f and i, j). Growth retardation can be observed after all treatments but 0.01 ppm Hg. This concentration, however, is characterized by a certain weak microscopic damage (see Table 1). No antagonistic effect of selenium could be observed when 1 ppm Se was added to the different concentrations of Hg.

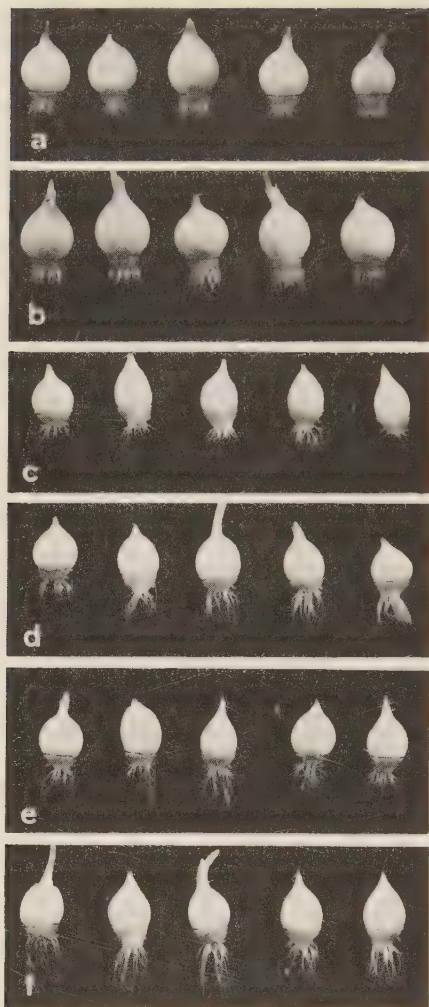


Fig. 4a-f. Growth retardation effects of different concentrations of Hg and fivefold excess of Se after 2 days treatment; a: 1 ppm Hg + 5 ppm Se; b: 0.4 ppm Hg + 2 ppm Se; c: 0.1 ppm Hg + 0.5 ppm Se; d: 0.04 ppm Hg + 0.2 ppm Se; e: 0.01 ppm Hg + 0.05 ppm Se; f: control water. Even excess of selenium gave no effect antagonistic to the toxic effects of mercury (eventually a weak additive effect only in b).

Table 2. Micromolar concentrations of MMC inducing similar responses in the original and the modified *Allium* tests

Response	Original test	Modified test
Loss of turgescence (lethality)	50	20
C-tumours	5	2
C-mitosis (highest frequency)	10	2-5
Microscopic disturbances	1	0.2

Times of treatment: 24 h in original test, 48 h in modified test; c-tumours about 5 days in both tests

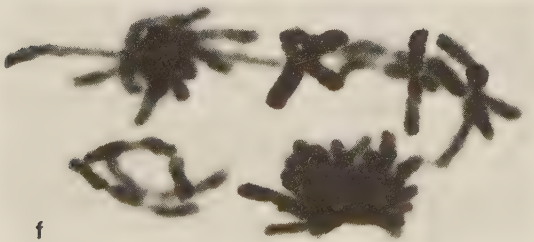
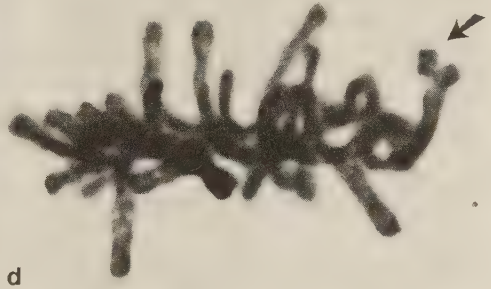
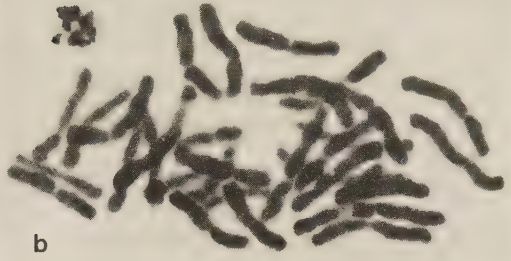
test are located 1 or 2 steps higher than those of the new, modified test. This result was perhaps to be expected, since in the original test the roots at the first examination had been exposed to the chemicals for 1 day only, whereas in the modified test they had been exposed for 2 days.

It may be an advantage that the modified *Allium* test needs lower concentrations to give specific response, which means that under certain conditions it is more sensitive than the original test. As mentioned above, this was an actual experience, when some highly diluted contaminants of natural waters were examined. The modified test is also especially well suited for photographic display of the macroscopic responses.

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Fig. 5a-g. Mercury-treated root tips of *Allium cepa* (5 days) and the characteristic c-mitotic response after treatment with Hg or with Hg + Se (2 days); a: root tips treated with 0.4 ppm Hg (about five times enlarged); b: c-anaphase (0.4 ppm Hg); c: c-metaphase (0.4 ppm Hg + 2 ppm Se); d: "normal" metaphase with fragment (arrow, 0.01 ppm Hg); e: disturbed anaphase with vagrant chromosomes, note the extended centromere region (arrow, 0.1 ppm Hg + 0.5 ppm Se); f: multipolar anaphase (0.1 ppm Hg + 0.5 ppm Se); g: micronuclei as a result of multipolar anaphase (0.4 ppm Hg + 2 ppm Se). Scale = 10 μ m.



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Two types of constitutive heterochromatin made visible in *Allium* by a rapid C-banding method

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On the basis of current C-banding methods — mainly in plants but also in mammals — a brief procedure has been worked out for root chromosomes in *Allium*. This procedure follows largely the schedule of SARMA and NATARAJAN (1973) for rye chromosomes. The results in *Allium* were better without treatment with $\text{Ba}(\text{OH})_2$, and also without NaOH , as observed by SCHWEIZER (1973) in plant material and by MCKENZIE and LUBS (1972) in human material. Furthermore, in *Allium* an additional step of hydrolysis in the schedule was favorable. Differences in response between two types of C-heterochromatin — centromeric and telomeric — were found to depend on conditions during storage of the slides before staining, mainly on the length of the period of storage.

C-banding in *Allium cepa* has been performed earlier by GREILHUBER (1972) and by STACK and CLARKE (1973a, b).

Materials and methods

Two *Allium* forms were examined: *Allium cepa* and the vegetatively reproduced hybrid *Allium proliferum*.

Root tips were grown in tap water for two or three days to a mean length of 2–3 cm. Before fixation in methanol/glacial acetic acid 3 : 1, different onions were treated for two hours with either 0.1 % colchicine or with methyl mercury chloride in the concentrations 20, 50 and 100×10^{-6} M which were shown earlier to yield good metaphase chromosomes with darker staining in centromeric and telomeric regions (FISKE-SJÖ 1969).

The fixations were kept in the cold ($+4^\circ\text{C}$) for 1–3 days and then hydrolysed in 0.1 N HCl at 60°C for 5 minutes, macerated and squashed in 45 % HAc, one root rip on each slide. The slides were almost directly placed on dry ice, the coverslips removed and the slides rinsed for some minutes in absolute alcohol and air dried overnight. The unstained slides were kept either in untight cardboard boxes or in tightly closed plastic boxes.

After different duration of storage staining for C-bands was performed as follows:

Slides were placed in 0.2 N HCl (denaturation) for 30 minutes in room temperature, rinsed in deionised water, placed in $2 \times \text{SSC}$ for 15 minutes in $+60$ – 66°C (renaturation), rinsed again in deionised water and stained for 10 minutes in Giemsa (Merck) diluted 1 : 20 in phosphate buffer with pH 7.1–7.2, again rinsed in deionised water and lastly air dried before examination in the microscope.

Observations and discussion

(1) Different types of C-bands

The main result of the present investigation is that the best C-bands are obtained, generally speaking, when staining is performed after a storage period of two weeks to two months. In the first hours and up to some days after preparation of the slides no bands at all will be seen. Good bands may be obtained in some cells much earlier than two weeks, especially when the chromosomes are squashed out of the cells.

Attempts were made to obtain bands earlier after preparation by prolonging the denaturation and renaturation times to one hour each. Thus weak bands resulted, but this method usually gave weaker bands than the shorter modification.

Two types of bands will appear when the present modification for C-banding is followed: Telomeric and centromeric heterochromatin, referred to as t.h.- and c.h.-bands, respectively. Making these two types of constitutive heterochromatin visible seems to depend on the length of the period of storage. During the first 24 hours most of the cells show chromosomes completely without bands of any sort (Fig. 1a, *A. proliferum* and 1b, *A. cepa*). After two days, weak t.h. bands begin to appear in some cells, but most of the cells contain chromosomes quite uniformly stained. After three days, most of the slides contain some cells with t.h.-banded chromosomes but most cells are still without bands. Rather

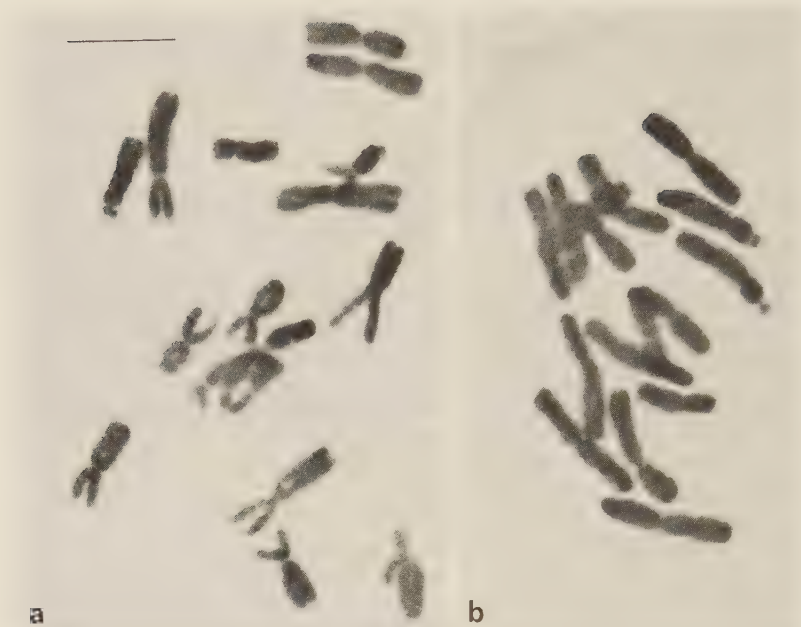


Fig. 1a and b. Chromosomes stained by the C-banding method directly after preparation. No bands are visible. a: *A. proliferum*; b: *A. cepa*. Scale = 10 μ .

good bands are seen in *A. proliferum*, in which bands regularly appeared earlier than *A. cepa*. After a week many cells still have chromosomes without bands, but now good t.h.-bands and even weak c.h.-bands appear in *A. proliferum*, whereas in *A. cepa* only weak t.h.-bands are seen (Fig. 2). After 2–3 weeks c.h.-bands are more frequent (Fig. 3). Now also *A. cepa* exhibits both types of bands, but here the bands usually are less distinct than in *A. proliferum*.

Up to about 2–3 months of storage both types of bands will weaken. In some cases the t.h.-bands will disappear completely after about three months. Fig. 4 shows chromosomes with almost no t.h.-bands left whereas the c.h.-bands are clearly visible in the primary and secondary constrictions.

The batch of slides which gave chromosomes of the type shown in Fig. 4 were kept in a cardboard-box which did not completely exclude air-

moisture. Slides stored in air-tight plastic boxes for three to four months never yielded cells of this type but showed rather good bands, both t.h.- and c.h.-bands. Experiments with storage in different types of boxes will be continued. ÖSTERGREN (pers.com.) has suggested that the plastic boxes used possibly release a compound with fixing effect on the material.

Different resistance to loss of chromatin stain from the two types of heterochromatin, centromeric and telomeric, was observed by LEVAN (1946, 1949): "The chromosome ends and the centromeres keep the stain even after the rest of the chromosomes are completely destained. Of these two types the centromeric heterochromatin is the more resistant" (l.c. 1949, p. 330). This behavior was found after fixation of the root tips in salts of heavy metals.

Treatments, such as killing with toluene (ÖSTERGREN 1947) or with chloroform (STEINEG-

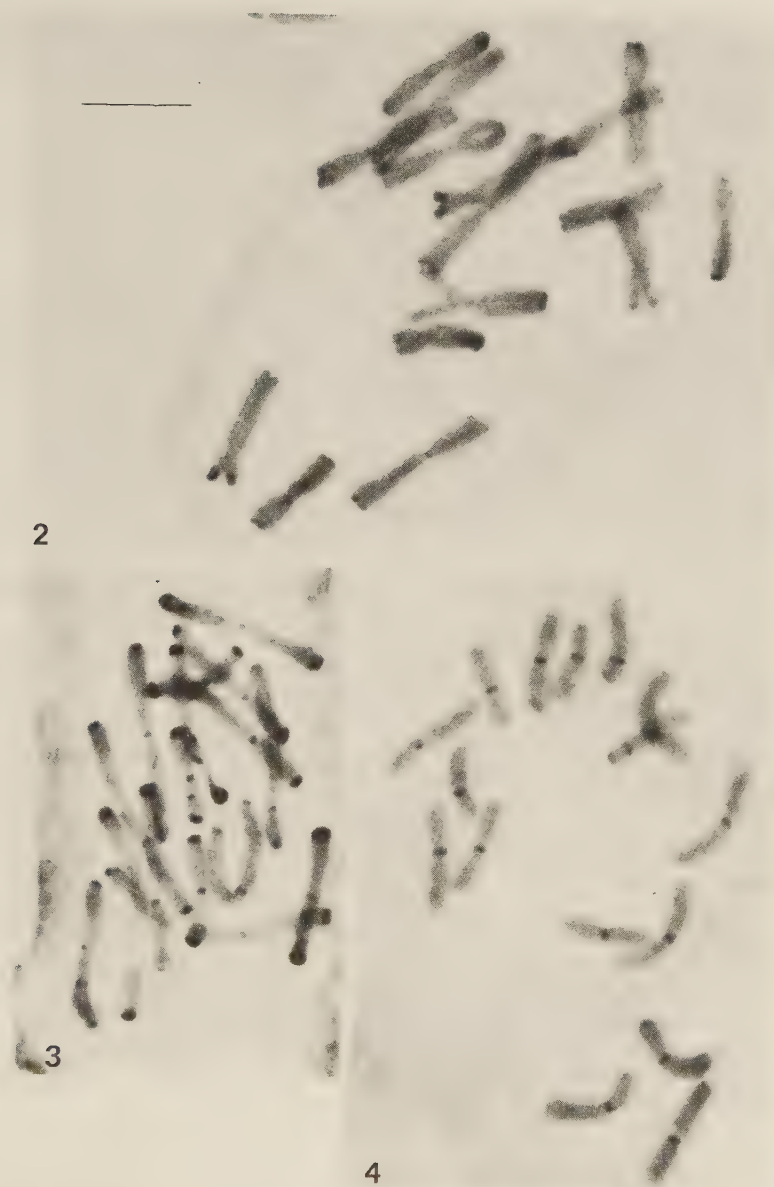


Fig. 2—4. C-banding. — Fig. 2. Two days old slide. Almost only telomeric bands are seen. *A. cepa*. — Fig. 3. Two weeks old slide. Both telomeric and centromeric bands. *A. proliferum*. — Fig. 4. Three months old slide. Bands only in primary and secondary constrictions. *A. cepa*. Scale = 10 μ .

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GER and LEVAN 1947) sometimes led to complete dissolution of the chromosome arms, only the centromeric regions being left.

Even though the background of the differences in appearance of C-bands is not yet quite clear, it is obvious that there are two types of constitutive heterochromatin in *Allium* chromosomes which show differential behavior to staining: the telomeric type at the ends of the chromosomes, and the centromeric type at the primary and secondary constrictions. Whether the heterochromatin at the secondary constrictions always behaves as the centromeric heterochromatin or represents a third type is still an open question.

Summing up, newly made slides give no bands at all; after some days telomeric bands appear. Both telomeric and centromeric bands may be seen during a period ranging from a few weeks to several months, and, depending on the method of storage, both types of C-bands or only the centromeric type of bands will be left after 3–4 months.

It was observed that at the time when the chromosomes give good bands, they are more difficult to karyotype (compare for instance, Fig. 1a, 3). The banded chromosomes are more variable in length indicating that they are more sensitive to squashing in 45 % HAc than in the 2 % orcein in 60 % HAc as in the ordinary schedule. Continued work may remove these shortcomings.

The C-banding of the *Allium* forms studied here shows almost no intercalary bands on the chromosomes; therefore G-banding will be needed for the study of the relationship between chromosomes of different species. Such work is under way.

(2) Effect of the mercury compounds on band formation

When slides were newly squashed and air dried, it was impossible to induce bands by any mercury treatment; neither did the mercurials interfere noticeably with band formation in older slides. Different mercury compounds may, however,

induce C-banding in *Allium* root cells. This was the case with mercury nitrate followed by crystal violet staining (LEVAN 1945) and methyl mercury chloride followed by orcein staining (FISKEJÖ 1969).

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Chromosomal relationships between three species of *Allium* as revealed by C-banding

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FISKESJÖ, G. 1975. Chromosomal relationships between three species of *Allium* as revealed by C-banding. — *Hereditas* 81: 23—32. Lund, Sweden. ISSN 0018-0661. Received May 14, 1975

A modification of the C-banding method yielded intercalary bands in certain chromosome pairs of *Allium fistulosum* and *Allium cepa*. One member of each pair of these identifiable chromosomes was present in the vegetatively propagated garden form *Allium proliferum*, indicating that *A. proliferum* represents a spontaneous hybrid between *A. fistulosum* and *A. cepa*. The chromosomes of this hybrid are compared with the chromosomes of the known hybrid artificially produced between the same two *Allium* species. Storage times of the slides, necessary for the appearance of the different types of bands are discussed.

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The C-bands obtained by different methods are usually found in the telomeric and centromeric regions. In some animals and in many plants also intercalary bands appear after different C-banding procedures. The nomenclature of these intercalary bands in plant materials is not yet standardized. Using a variety of plant materials and applying different Giemsa staining techniques that comprise acid treatment and incubation in $2\times$ SSC, SCHWEIZER (1973) obtained different bands including intercalary ones and referred to the banding as "Giemsa staining of H-segments". DÖBEL et al. (1973) used the term Giemsa bands for the different bands obtained in *Vicia faba* by a method modified from BERGER (1971) and involving urea treatment. In animal materials treatment with urea gives rise to G-bands (KATO and MORIWAKI 1972), which mostly do not show up with C-banding methods. BURGER and SCHEUER-MANN (1974), using a C-banding modification of the C-banding techniques described by SUMNER (1972) for the differential staining of *Vicia faba* chromosomes, distinguished between two types of bands: C-bands (in centromeres and secondary

constrictions) and G-bands (intercalary bands). These latter agree with those obtained in *Vicia faba* by DÖBEL et al., using urea. SCHWEIZER (1974) also applied a modification of SUMNER's method (1972) to C-banding of different plant chromosomes. Thus, in *Secale cereale* he observed terminal, intercalary and centromeric bands, all referred to as C-bands. He pointed out that "the Giemsa banding techniques that have been developed for animal and human chromosomes are in general not directly applicable to plants".

The present work is also based on a modification of SUMNER's methods (1972), and all types of bands (in telomeres, in primary and secondary constrictions as well as in intercalary locations) will be referred to as C-bands, even though the same bands will also occur with methods involving the use of urea or trypsin. These latter methods have not contributed any additional bands, however.

So far, no reports have been published on G-banding in *Allium* but some authors have described C-banding patterns (GREILHUBER 1972; STACK and CLARKE 1973a, b; FISKESJÖ 1974). Differential

staining of the heterochromatin of kinetochores and nucleolus organizers was performed in *Allium cepa* by STACK (1974) and a special Hy-banding technique for heterochromatin in *Allium cepa* and *A. flavum* by GREILHUBER (1974). By means of a fluorescence method VOSA (1971) established a certain banding pattern in *Allium carinatum*.

In *Allium* the C-banding treatments reported usually reveal telomeric bands, and occasionally also centromeric bands. In the present work, however, all three types of C-bands are demonstrated in three *Allium* forms, viz. in centromeres, in secondary constrictions and in other intercalary locations.

Materials and methods

Three forms of *Allium* were examined: *Allium fistulosum*, *A. cepa*, and the vegetatively reproduced hybrid *A. proliferum*, all with $2n=16$.

The C-banding was performed by the rapid method described earlier (FISKESJÖ 1974): After denaturation in HCl, renaturation with $2\times$ SSC but without exposure to $\text{Ba}(\text{OH})_2$, staining was performed with Giemsa. In order to obtain both telomeric and centromeric bands, it was of main importance to store the slides for at least one week prior to the C-banding treatment.

Methods for G-banding were also tried, namely modifications of the urea method (DÖBEL et al. 1973; HANSEN-MELANDER et al. 1974) or the trypsin method (WANG and FEDOROFF 1972). None of these methods gave stainings of good quality in the *Allium* material, and any bands appearing were in the same position in the chromosomes as those obtained by the C-banding modification used. Of the G-banding methods, the best results were obtained by the 3–4 hours urea treatment used for rabbit chromosomes by HANSEN-MELANDER et al. (1974). In *Allium* this treatment resulted in distinctly stained telomeric and centromeric regions but with too little contrast between chromosomes and cytoplasm. Sometimes weak intercalary bands were discernible, and these bands also had the same position as the intercalary bands obtained by the C-banding method.

Q-bands were also studied according to the modification of QM-staining by MANOLOV et al.

(1971); by this procedure telomeres and satellites became clearly visible as negatively stained regions, but no intercalary bands appeared.

Observations and discussion

1. C-bands and the identification of individual chromosome pairs

In conventional stainings one chromosome pair, the satellited one, can be identified easily both in *A. fistulosum* and *A. cepa*. After C-banding the satellited pair shows characteristic bands, and in addition three more pairs in *cepa* and two in *fistulosum* become identifiable. Of these latter, two pairs in each species exhibit intercalary bands, and the third pair in *cepa* has a characteristic telomeric banding, only one end of the chromosome being stained (Fig. 1, 2). Still another pair in *cepa* may sometimes be recognized by its very faint telomeric bands at both ends. Since, however, the staining of the telomeres of this chromosome is variable, this last identification is uncertain. In contrast to conditions in *fistulosum*, the *cepa* chromosomes are generally characterized by their rather weakly stained telomeres. The four telomeres of this chromosome pair together with the two telomeres that lack bands in the pair mentioned before make six unbanded telomeres in a somatic cell of *A. cepa*. This is in agreement with the counts of C-banded telomeres in interphase cells of *A. cepa* performed by STACK and CLARKE (1973b). These authors never found more than 28 C-banded telomeres of 32 possible, and usually considerably fewer.

The satellite chromosomes of *A. fistulosum* and *A. cepa* will be referred to as s_1f and s_1c (LEVAN 1936) and the other identifiable chromosomes with median to submedian centromeres as m_1f , sm_2f and sm_1c , m_2c , m_3c .

The characteristics of the identifiable chromosomes are as follows (s =satellite chromosome, m =median centromere, sm =submedian centromere, f =*fistulosum*, c =*cepa*):

s_1f . — The satellite chromosome of *A. fistulosum* is easily identifiable and has a big satellite at the short arm. This satellite as well as the telomere of the long arm and the primary and nucleolar constrictions are very darkly stained. Sometimes a band is seen near the centromere in the long arm



Fig. 1a—c. — a: *Allium fistulosum*, three pairs of identifiable chromosomes (arrowheads); b, c: *A. cepa*, four pairs of identifiable chromosomes (arrows). Note the tendency to somatic pairing.



Fig. 2a—c. The hybrid *Allium proliferum*; a: identifiable chromosomes from the parental species *A. fistulosum* (arrowheads) and *A. cepa* (arrows); b: 8 chromosomes with darkly stained telomeres (from *fistulosum*) and 8 chromosomes with less densely stained telomeres (from *cepa*), even some intercalary bands may be seen; c: in this cell s_{1f} (from *fistulosum*) and s_{1c} (from *cepa*) without satellite are clearly seen, also m_{3c} and m_{1f} are easily identifiable.

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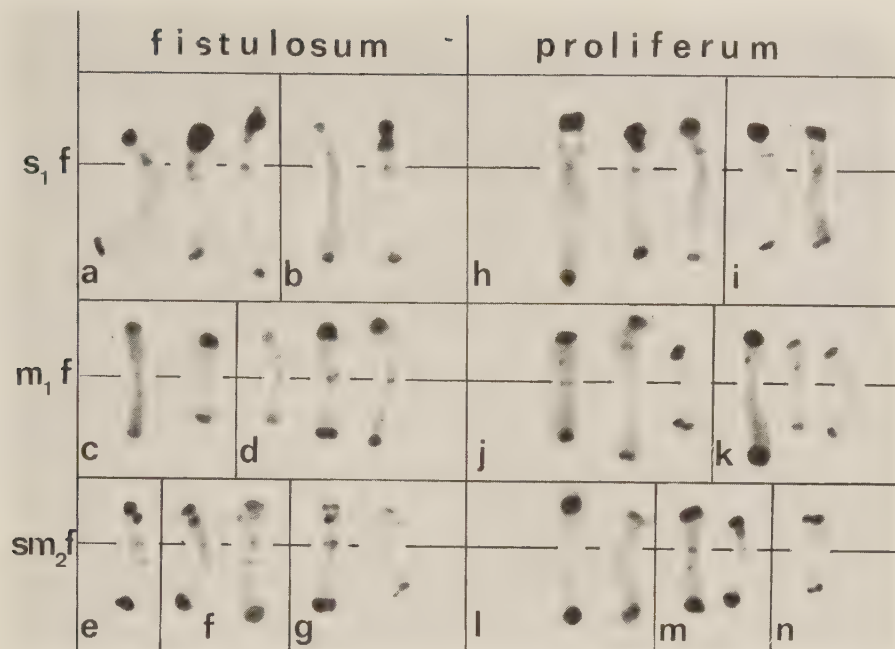


Fig. 3. Examples of the identifiable chromosomes of *A. fistulosum* taken from different cells. The appearance of these chromosomes is about the same in *fistulosum* (a—g) as in the hybrid *proliferum* (h—n). The same magnification as in Fig. 4.

(Fig. 3a, h), but sometimes this band is not stained (Fig. 3b, i). This band almost always appears over the entire width of the chromosome. The satellite is often removed from the chromosome by squashing, but a thin threadlike connection is always visible between the satellite and the telomere.

m₁f. — This is a rather small chromosome with almost median centromere. It has one intercalary band located in the middle of the shorter arm. This band is very often visible as two adjacent dots (Fig. 3c, j), but sometimes only one dot is visible (Fig. 3d, k). Sometimes only one of the homologues shows this band, and sometimes the band is weaker in one homologue than in the other, but usually it is found in both homologues of this pair. In exceptional cells this is the only intercalary band visible (even though, in the same cell, both telomeric and centromeric bands are visible).

sm₂f. — This chromosome is submetacentric and the smallest chromosome of *A. fistulosum*. Two intercalary bands may appear — one located in the short arm near the telomere (Fig. 3e, f, g, l, m, n) and one in the long arm near the centromere (Fig. 3e, f, l, m). The former one is the most darkly stained intercalary band in *A. fistulosum* and is always visible as two adjacent dots. It is the first intercalary band to appear, and it may be visible even at the time when the telomeric bands appear. Among all the intercalary bands, this has the best resistance against storage. Both in *fistulosum* and in the hybrid this is often the only intercalary band visible.

The intercalary band in the long arm is the most variable one. It may be completely absent (Fig. 3g, n) or appear either as two dots (Fig. 3e, l) or as a band (Fig. 3f, m).

s₁c. — The satellite chromosome of *A. cepa* is easily identifiable being the only subtelocentric

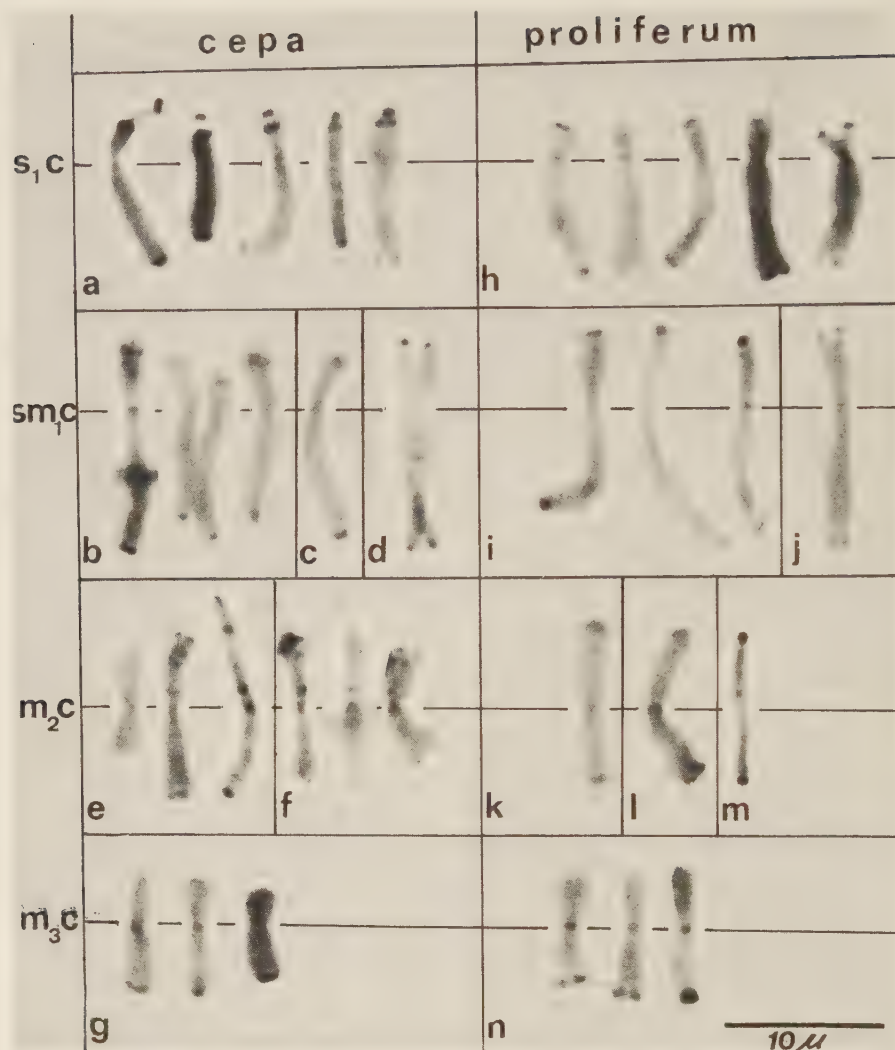


Fig. 4. Examples of the identifiable chromosomes of *A. cepa* taken from different cells. The appearance of these chromosomes is about the same in *cepa* (a—g) as in *proliferum* (h—n).

chromosome of the genome. It possesses a very small satellite at the end of the short arm. The satellite, as well as the telomeric end of the long arm and the primary and nucleolar constrictions, are darkly stained in C-banding (Fig. 4a). Only very seldom it happens that the satellite thread is removed at some distance from the short arm by the squashing.

sm₁c. — This chromosome is one of the longest with submedian centromere in *A. cepa*. One intercalary band near to the end of the short arm is always present in both homologues. It usually appears as a thin band (Fig. 4c, d) or as two adjacent dots, one in each of the sister chromatids (Fig. 4b, d, j), and sometimes as only one dot (Fig. 4i). When this band is visible, very often the adjacent telomeric band disappears (Fig. 4b, c), but the telomere of the longer arm is always stained.

m₂c. — A medium-sized chromosome with almost median centromere. In the shorter arm there are two intercalary bands, one in the proximal and one in the distal third of the arm. They appear as two adjacent dots (Fig. 4e, k, l), or sometimes only a dot in one of the chromatids (Fig. 4f, m). In the long arm not far from the end, a weak intercalary band often appears as one small dot in each chromatid (Fig. 4e, k); but this band, however, often fails to show up (Fig. 4f, l, m). If these three bands are visible, they always appear in both homologues. Both telomeres are always stained.

m₃c. — A small metacentric chromosome. It has no intercalary bands but the telomere of the shorter arm is very weakly stained or not stained at all. The other telomere seems to be a little more stained than the other *cepa* telomeres (Fig. 3g, n).

2. Time of the appearance of the bands

The response to the C-banding is different in the three *Allium* forms. In *A. fistulosum* the telomeric bands appear already one day after the preparation of the slides, and the intercalary bands may become visible shortly after the telomeric bands, especially the dark band in the short arm of *m₂f*. Compared to *A. fistulosum*, C-banding in *A. cepa* always requires a longer storage time. Two days are a minimum for the telomeres to appear, and

the intercalary bands usually do not become visible until after a few weeks.

The C-bands are weaker in *A. cepa* than in *A. fistulosum*. These differences in stainability of the chromosomes of the two *Allium* species are maintained in the hybrid. This may be best observed in Fig. 2b.

Sometimes only the intercalary bands of the identifiable chromosomes from *fistulosum* are visible in the hybrid (Fig. 2c).

In a previous report dealing with the same material (FISKEŠJÖ 1974), it was stated that different storage time of the slides was necessary for bringing out the different types of bands. Directly after the preparation of the slides no bands at all were seen. After some days, the telomeric bands started to appear, and after some longer storage also the centromeric heterochromatin became visible. In the present work these results could be repeated, except for the observations that only centromeric banding was detectable after long storage in a not airtight cardboard box. Repetition of these experiments indicated that after storage for three months or more, the centromeric bands improved in stainability and became more distinct than the telomeric bands. After storage for half a year or more the whole chromosomes were faintly stained, and the bands at telomeres and primary and nucleolar constrictions showed very little contrast in relation to the rest of the chromosomes and to the darkly stained cytoplasm.

In the present material the time of appearance of the intercalary bands is somewhat variable, but when the different bands appear they will always be visible at specific sites of the chromosome. Sometimes there are no intercalary bands at all, especially in newly made preparations, but throughout the storage time it happens that the intercalary bands fail to appear even though the telomeric or both the telomeric and centromeric regions become stained. The intercalary bands do not appear until after the slides are old enough for telomere staining, but most often they appear before the slides are stored long enough for centromeric staining.

For the present purpose of distinguishing between the three *Allium* forms, only telomeric and intercalary bands are of importance.

The banding results differ slightly between batches of slides and also between occasions of staining. The mode of previous cultivation of the

onions, fixing time, storage temperature and other variable factors may interfere with the results of the C-banding treatment. It may be recommended to keep the slides for staining in tightly closed plastic boxes up to half a year, or more if the plastic boxes are only rarely opened.

3. Relationships between the three *Allium* forms

It was proposed by LEVAN (pers. comm.) that *A. proliferum* is a hybrid between two *Allium* species, most probably *A. cepa* and *A. fistulosum*. KIHLMAN (1971) uses the name *A. proliferum* for this vegetatively reproduced onion, which has earlier been called the tree onion or the Egyptian onion. According to him it is "classified as a vegetatively reproduced form of *Allium cepa* (*A. cepa* var. *proliferum*), although some authors claim that all proliferous forms belong to *Allium fistulosum* (for references, see JONES and MANN 1963)".

Concerning the only satellite chromosome of *Allium proliferum* KIHLMAN writes: "This satellite chromosome is, in fact, very similar to that of *A. fistulosum*, which supports the suggestion that the tree onion is more closely related to *A. fistulosum* than to *A. cepa*."

The present observations on the identifiable chromosome types of *A. fistulosum* and *A. cepa* support the interpretation of *A. proliferum* as a true spontaneous hybrid between these two species. The three identifiable chromosome types of *A. fistulosum* and the four of *A. cepa* are all found in the hybrid (Fig. 2a), and no other chromosomes with intercalary bands were seen. With one exception these chromosomes have the same appearance in the hybrid as in the parent species. This exception is the satellite chromosome of *A. cepa* which has no satellite in the hybrid (Fig. 4h and 2c). Since this chromosome is very similar in size and shape to the s_{1c} (Fig. 4a), it is tempting to consider it as an s_{1c} without a satellite. This assumption is compatible with observations of the nucleoli in the three forms: interphase cells of *proliferum* generally contain only one nucleolus, whereas in *fistulosum* and *cepa* they contain one or two nucleoli. Counts in 100 cells of each form gave the following average numbers of nucleoli per cell:

<i>fistulosum</i>	1.52
<i>cepa</i>	1.50
<i>proliferum</i>	1.06

It should be observed that the 6 nuclei of *proliferum* recorded as having two nucleoli were somewhat uncertain; also one of them may have been tetraploid, judging from its size.

The chromosomes of *A. proliferum* which are not individually distinguishable are similar to the rest of the chromosomes of *A. fistulosum* and *A. cepa*. In the three hybrid cells pictured (Fig. 2a, b, c) one may recognize all the characteristics of *A. proliferum* chromosomes. In Fig. 2a all the identifiable chromosomes are seen except the satellite chromosome of *A. cepa*. In Fig. 2b the differences in telomeric staining between *fistulosum* chromosomes (strongly stained telomeres) and the *cepa* chromosomes (weakly stained telomeres) are quite obvious.

An artificial hybrid between *A. fistulosum* and *A. cepa* was produced by LEVAN (1936). The chromosomes of this hybrid (LEVAN 1936, Fig. 1j-y) were very similar to the chromosomes of *A. proliferum* reported here (Fig. 2, 3, 4). In the artificial hybrid, however, the s_{1c} still had the satellite. This was also the case in the polyploid offspring of the hybrid (LEVAN 1940). In the artificial hybrid there was a great amount of meiotic disturbances and very few cells with 8 bivalents. Other investigators (EMSWELLER and JONES 1935; MAEDA 1937) found higher degree of complete bivalent pairing in F_1 between other parental lines, but all hybrids had a high degree of sterility. *A. proliferum*, which may be considered a spontaneous hybrid between *A. fistulosum* and *A. cepa*, has very few flowers and is propagated by small bulbs in the inflorescences.

In a highly sterile species hybrid, such as *Allium cepa* × *fistulosum*, natural selection would be expected to act very strongly in favor of forms with even more efficient vegetative propagation and would reasonably end up with forms such as *A. proliferum*.

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Benzo(a)pyrene and N-methyl-N-nitro-N-nitrosoguanidine in the *Allium* test

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When a genotoxic chemical is studied in different test systems, the results are often in good agreement. In the case of polycyclic hydrocarbons, however, one may expect different results due to different metabolizing abilities of different cell types. The Chinese hamster cell line V 79 does not possess the mixed-function oxidase (MFO)-system necessary for metabolizing, for example, benzo(a)pyrene (BP), which has to be metabolically activated before exerting its biological effect. Other chemicals, as N-methyl-N-nitro-N-nitrosoguanidin (MNNG), are inducing effects even without the MFO-system. In the present investigations, MNNG gave similar results in V 79 and *Allium*, but BP gave clearly lesser effects in the *Allium* test. This means either that (1) *A. cepa* has certain amounts of the MFOs, but not sufficient for complete metabolism of the BP; (2) BP is not fully absorbed by the root cells, or (3) BP is not completely solved (in acetone/water). Whatever the reason, it may be concluded that there are certain restrictions for the use of the *Allium* test: Weak damage in the *Allium* test may correspond to more severe damage in other test systems.

Since pretreatment with MNNG or BP spoiled the chances to obtain good chromosome structures in subsequent C-banding, the ordinary orcein-squash technique was preferred.

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The importance of testing genotoxic chemicals in more than one test system is often pointed out. One of the main reasons for using several tests is to investigate whether different cell types differ in their sensitivity to environmental chemicals. Another reason is to gather information concerning the predictive value of short-term tests.

The two tests chosen for comparison in the present investigation are one plant test (the *Allium* test; *A. cepa*), and one test with mammalian cells (the V 79 test; a Chinese hamster cell line). Different results in these tests may mean that the two test organisms have different types of metabolism, whereas similar results would indicate similar metabolism.

As a routine, the *Allium* test includes a so-called negative control performed with tap water of known and "good" quality. The V 79 test includes two controls: one negative (with culture medium), and one positive; the latter varies depending on whether the cultivation of V 79 cells is combined with an extra metabolizing system or not: benzo(a)pyrene (BP) is used for the cell-mediated test (HUBERMAN and SACHS 1974), and N-methyl-N-nitro-N-nitrosoguanidine (MNNG), for the direct test.

Previous comparisons between the *Allium* and V 79 tests have comprised among others, certain mercury compounds (FISKESJÖ 1979a) and dichlorophenol. While the mercury compounds have given similar results in direct tests with both test systems, dichlorophenol has induced effects in direct tests with *Allium cepa* (FISKESJÖ et al. 1981) but has needed extra metabolic activation to give toxic effects in V 79 (FISKESJÖ, unpubl.)

Such differences in results probably depend mainly on various amounts of mixed-function oxidases (MFO). These are usually lost in established cell lines, as in the V 79 (NEBERT and GELBOIN 1968; NEWBOLD et al. 1977).

Whether plant materials may activate chemicals has only recently been taken up for discussion; however, several plant materials have been found to activate certain pesticides into genotoxins (PLEWA 1978).

Certain chemicals, such as polycyclic hydrocarbons (e.g., BP) and sometimes also monocyclic compounds (as dichlorophenol), often need the presence of the MFO-system to be biologically active.

While in the present experiments with *Allium* and V 79 MNNG is expected to cause toxic effects

directly (without MFO) in both test systems, and BP is known to require metabolizing in V 79, it is not fully known to what degree *Allium* cells have the capability to activate BP.

Material and methods

Biological test material. — Equal-sized small onions of a population of *Allium cepa*.

Chemical test material. — Benzo(a)pyrene (BP) (Merck); N-methyl-N-nitro-N-nitrosoguanidine (MNNG) (Sigma); acetone.

The concentrations to be tested (10, 5, 1, 0.5, $\mu\text{g/ml}$) were chosen below and above the concentration 1 $\mu\text{g/ml}$ of the positive controls in the V 79 tests (HUBERMAN and SACHS 1974; FISKESJÖ 1979a). The 5, 1 and 0.5 $\mu\text{g/ml}$ -solutions of both BP and MNNG were dissolved in 0.5 % acetone, and the 10 $\mu\text{g/ml}$ -solution was dissolved in 1 % acetone. Additional controls with 0.5 and 1 % acetone, respectively, were performed. BP apparently was soluble in the acetone solutions, but, after further dilution in water, precipitation was formed especially in the 1 % acetone solution, and the test solutions remained weakly opaque throughout the experiment.

Control medium and dilution liquid. — Normal tap water known to give good root growth (no iron pipes; no decalcification).

The detailed procedure has been described earlier (FISKESJÖ 1979b). Briefly, the test is based on series of onion bulbs from which the outer dry layers have been carefully removed and which are placed on top of test tubes containing the different

concentrations of the solutions to be tested and, in addition, one series of controls. The test liquids are changed every day until day 3; thereafter only evaporated amounts of liquids are replaced.

After 2 days of cultivation, one root tip from each onion is harvested for preparation of slides for cytological studies: Orcein-stained slides (FISKESJÖ 1975a, 1979b) or Giemsa-stained slides for chromosome banding (FISKESJÖ 1974).

After 5 days of cultivation, the series of onions are photographed and the mean lengths of roots measured ($\bar{x}$ = mean length of the root bundles within a series).

Results and discussion

1. Macroscopic effects

Results are most easily surveyed by observing and photographing the root lengths; a photographic "table" is shown in Fig. 1. Acetone gave by itself some growth retardation, especially the 1 % solution (Fig. 1i), but, compared to the acetone control, the 10 $\mu\text{g/ml}$ concentrations of both MNNG and BP showed clear additional effects (Fig. 1a and e). In this concentration, however, BP is not completely solved (see the opaque liquids in Fig. 1e). MNNG gave clear growth retardation both in the 5 $\mu\text{g/ml}$ concentration (Fig. 1b) and in the 1 $\mu\text{g/ml}$ concentration (Fig. 1c), while 0.5 $\mu\text{g/ml}$ only gave slightly shortened roots (Fig. 1d; cf. the 0.5 % acetone control, Fig. 1k). BP in the concentration of 5 $\mu\text{g/ml}$ gave somewhat shorter roots than the control (Fig. 1f), while 1 $\mu\text{g/ml}$ and 0.5 $\mu\text{g/ml}$ did not cause any reduction of the growth compared to the 0.5 % acetone control.

The same data on the growth of the roots are recorded in Table 1, in which it is easily seen from the mean root length values that while MNNG caused toxic effects in all concentrations tested, BP caused such effects only to a slight degree in the two highest concentrations tested.

Apparently, 0.5 $\mu\text{g/ml}$ MNNG gives a clear toxic effect in *Allium*. This concentration was the positive control in the V 79 tests and induced some 70 % reduction of survival. The effect of this concentration in *Allium*—below 10 % growth retardation—is less severe, which may be due to the instability of the MNNG in conjunction with the longer time of treatment in the *Allium* test.

In BP, the degree of effect differs even more between the two cell types: in the V 79 test, 1 $\mu\text{g/ml}$ of BP caused reduction of survival of some

Table 1. Mean root length values (n=10) after 5 days' treatment with different concentrations of N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and benzo(a)pyrene (BP)

Treatment $\mu\text{g/ml}$		Mean root length $\pm$ SD (in cm)
MNNG	10	1.10 $\pm$ 0.34
	5	1.77 $\pm$ 0.36
	1	5.57 $\pm$ 0.36
	0.5	6.40 $\pm$ 0.32
BP	10	2.91 $\pm$ 0.28
	5	5.58 $\pm$ 0.30
	1	6.67 $\pm$ 0.53
	0.5	6.57 $\pm$ 0.20
Acetone	1 %	4.95 $\pm$ 0.46
	0.5 %	6.85 $\pm$ 0.57
Control (tap water)		7.18 $\pm$ 0.50

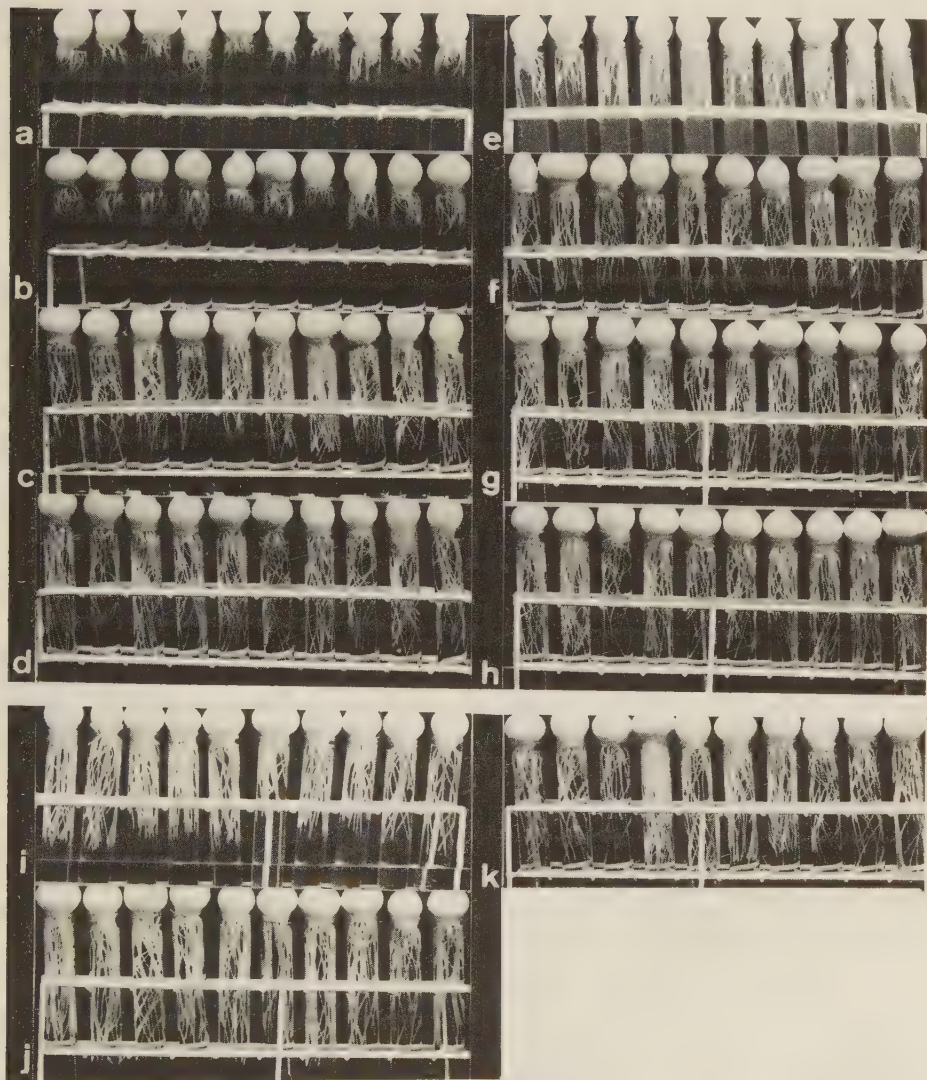


Fig. 1a-k. Onion series after 5 days of treatment with MNNG (a-d), BP (e-h), controls (i-k). a and e 10 $\mu\text{g/ml}$. b and f 5 $\mu\text{g/ml}$. c and g 1 $\mu\text{g/ml}$. d and h 0.5 $\mu\text{g/ml}$. i 1 % acetone. k 0.5 % acetone. j tap water.

50 %, while in the *Allium* test, the two lowest concentrations, 1 µg/ml and 0.5 µg/ml had no obvious effect on growth at all.

2. Microscopic effects

Incidence of deviations

The microscopic effects are listed in Table 2. MNNG interferes with all types of parameters studied. The *mitotic index* was clearly correlated to the root length, thus increasing with decreasing concentrations of the treatments. The *number of mitoses analyzed* reflected the condition of the root tips: more mitoses were available in lower concentrations. *Normal metaphases* were somewhat fewer in all treatments than in the controls. *Normal anaphases* were lowered in concentrations higher than 0.5 µg/ml, while *delayed anaphases* were markedly higher only in the concentration 5 µg/ml. Stickiness was only scarcely occurring from the MNNG treatment. Chromosome fragments and bridges were much more frequent than in the control.

This means that chromosome breakage is the most typical effect of MNNG, which is in good accordance with the mutagenic effect of this compound. The incidence of full *c-mitosis* and weaker *c-mitotic* effects, as *vagrant chromosomes*, *hyperdiploid* or *polyploid mitosis*, (*Mitoses* > 2n), was only slightly increased, indicating that MNNG acts directly on the chromosome material and only to a little extent on the spindle mech-

anism. In the last column of the table, observations of *micronuclei* during interphase are listed, showing the expected result: the higher the number of chromosome breaks, the higher the number of micronuclei.

The microscopic effects of BP are listed in Table 2 in the same way as those of MNNG. Since a preliminary scanning of the two lower concentrations of BP showed no clear deviations from normality, they were excluded from the microscopic screening. *Mitotic index* was somewhat lowered in the higher concentrations (10 and 5 µg/ml); the number of *delayed anaphases* was slightly higher than in the control, indicating that the mitotic rate was tardier. *Normal metaphases* were a little fewer than in the control, and there was more stickiness. This was actually the most typical toxic effect of BP. Other parameters did not deviate substantially from the control, but even a great part of the so-called normal stages were bad-looking, probably indicating that the cells were under some toxic influence from the treatment.

Types of deviation

Instances of the strong chromosome breaking activity of MNNG are presented in Fig. 2, namely fragments of different sizes in a-d, one pseudochiasma and a big double fragment in d, the frequent bridges between anaphase chromosomes in a and d, etc. In the metaphase of Fig. 2c, one of the rather few chromatid interchanges between two chromosomes may be seen, and in the inter-

Table 2. Cytological effects of treatments with different concentrations of N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and benzo(a)pyrene (BP). The µg/ml solutions correspond to 6.8×10^{-5} M (MNNG) and 4×10^{-5} M (BP), respectively.

For microscopic studies, slides were prepared from each of 5 bulbs from a series of 10; with one root tip for each slide. The mitotic index was based upon 1,000 cell counts (200 cells from each slide), and for the classification of cytological effects, some 500 cells were scored (about 100 cells per slide)

	MNNG, µg/ml				BP, µg/ml				Acetone		Control ^a
	10	5	1	0.5	10	5	1	0.5	1 %	0.5 %	
Mitotic index	0	0.3	2.9	4.7	3.7	4.9	5.5	5.2	4.3	5.1	5.5
No. cells analyzed	13	51	264	500	500	500	b)	b)	500	500	500
Normal metaphases	46.1	37.3	45.4	48.2	52.0	44.8			54.8	56.0	61.2
Normal anaphases	15.4	7.8	15.9	25.4	28.7	31.6			30.2	29.0	25.4
Delayed anaphases	(0)	25.5	6.4	9.8	9.8	11.8			10.6	9.6	7.8
Stickiness	(0)	0	3.4	1.4	6.7	10.4			2.4	3.0	2.4
Bridges	(0)	2.0	5.7	3.8	1.0	0			0.4	1.6	1.8
Fragments	(15.4)	17.6	18.6	7.2	0.5	0.2			0.2	0.4	0.2
C-mitosis	(23.1)	2.0	1.5	3.0	0.8	0			0.4	0.2	0.6
Vagrant chromosomes	(0)	3.9	2.6	1.2	0.5	1.2			1.0	0.2	0.2
Mitoses >2n	(0)	3.9	0.4	0	0	0			0	0	0.4
Micronuclei	+	+	++	+	(+)	(+)			(+)	-	-

a) Tap water

b) Not analyzed

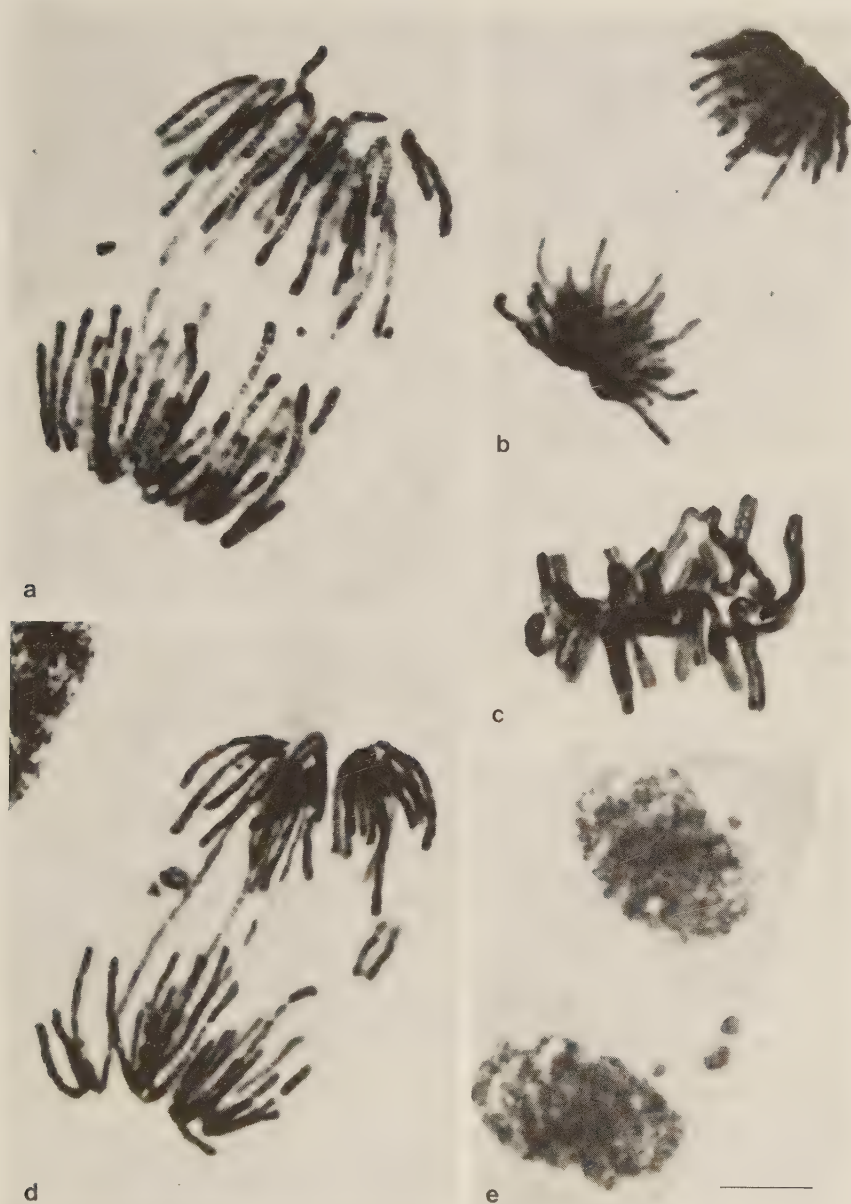


Fig. 2a-e. Chromosome breaking effects of MNNG. **a** Bridges and fragments. **b** Small fragments. **c** Chromatid breaks. **d** Bridges, big fragments and spindle disturbances. **e** Micronuclei. (a-d: 1 μ g/ml; e: 0.5 μ g/ml). Bar: 10 μ m.

phase of Fig. 2e the fragments reappear as micronuclei.

In Fig. 3e, a characteristic effect of BP on metaphase chromosomes is shown in comparison with the normal metaphase and anaphase chromosomes of Fig. 3a and b. The most typical microscopical effects of BP were sticky and bad-looking chromosomes. Being mutagenic in other test systems, for instance in the cell-mediated test with V 79 cells (HUBERMAN and SACHS 1974), BP would be expected to cause chromosome breaks with consequent formation of micronuclei, but such effects were seen only exceptionally. This may be due to several reasons: the BP is dissolved only incompletely; it is uncertain how much of the compound penetrates through the cell wall; and eventually, when inside the cell, the BP may not be subject to complete metabolizing by MFO.

Whatever the reason, the following two conclusions can be drawn: (1) The *Allium* test—on plant material—is obviously capable of metabolizing BP to some extent. This indicates that the *Allium* cells are provided with MFO activity. (2) The finding even of weak genotoxic effects at a first screening within a program for control of environmental risks must prompt continued studies warranting highest chances possible to reveal any kind of contaminant.

3. Use of chromosome banding technique

Attempts were made to study the effects of MNNG and BP with the modification of the Cbanding technique applied to *Allium* chromosomes by FISKESJÖ (1974, 1975b).

Since this staining procedure is known to be very sensitive, it was not surprising that pretreatments with MNNG or BP before the staining gave unsatisfactory results. Especially after treatment with higher concentrations (10 and 5 $\mu\text{g/ml}$) of MNNG and BP, both chromosomes and cytoplasm took a dark colour and the chromosomes were sticky and completely lacking the normal "crisp" structure without which no bands become visible in the chromosomes.

After some trials, it was ascertained that a mild colchicine treatment (0.01 % for 6 h) before weak treatments with MNNG or BP led to some improvement of the chromosome structures. Fig. 4a and b are such metaphases treated with 1 $\mu\text{g/ml}$ of MNNG or BP, respectively. In both, chromosome structures are sticky and evidently badly affected by the toxic action of the compounds. Especially in 4b (treatment with BP), terminal C-bands, and

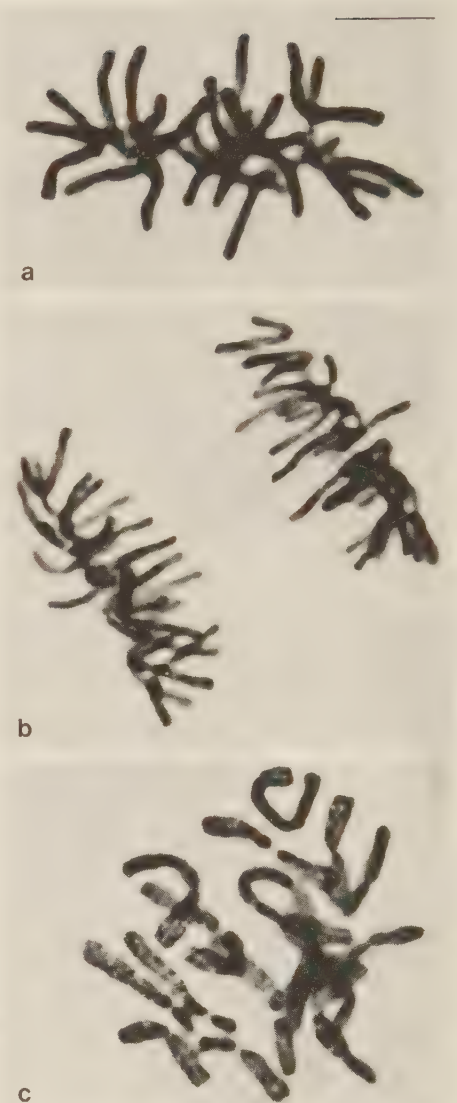


Fig. 3a-c. Fixation and stickiness-effect of BP in comparison to normal stages. a Normal metaphase, b Normal anaphase. c BP 5 $\mu\text{g/ml}$. Bar: 10 μm .

also some intercalary ones are discernable. That they are not comparable to good C-bands in metaphases, not exposed to MNNG or BP treatment, is

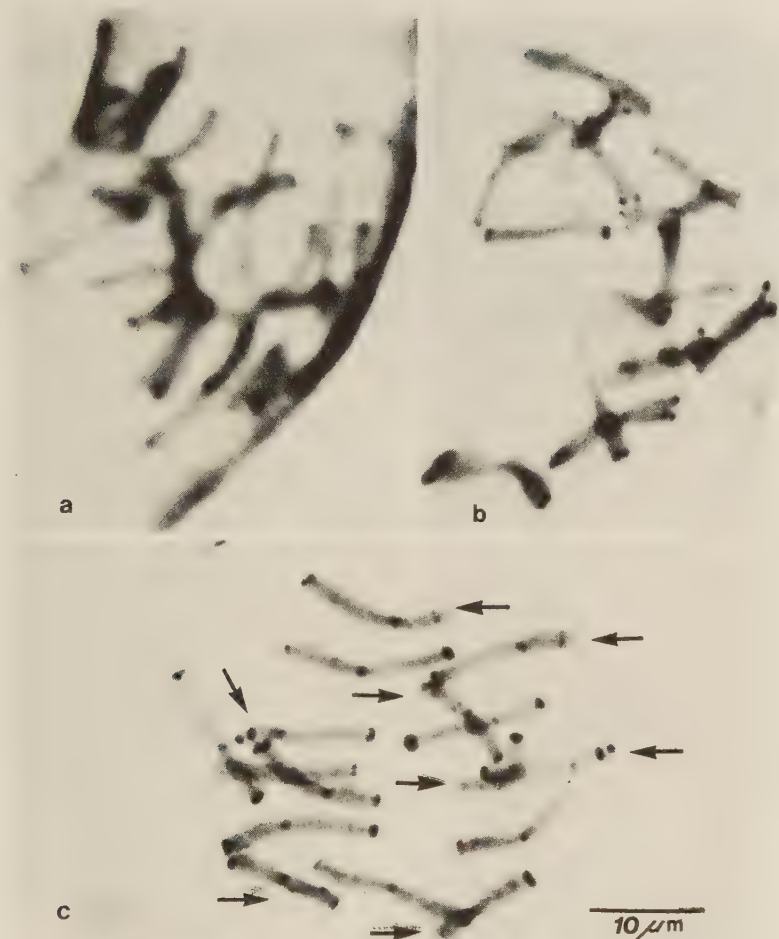


Fig. 4a-c. C-banded chromosome groups of *Allium cepa* after treatments with a MNNG 1 µg/ml and 0.01 % colchicine, b BP 1 µg/l and 0.01 % colchicine, c only 0.1 % colchicine. Note the 4 pairs of identifiable chromosomes (arrows). (c from FISKESJÖ 1975b).

clear from Fig. 4c, which pictures one such metaphase with good C-banding; easily permitting the identification of 4 chromosome pairs with characteristic patterns of C-bands. It was concluded that for the purpose of rapid screening of chromosome damage induced in *Allium* roots by MNNG and BP, ordinary orcein squashes were much to be preferred.

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